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August 8, 2012

Dr. Lisa Brines
National List Manager, Standards Division
USDA/AMS/NOP, Standards Division
Attention: Stacy Jones King
1400 Independence Ave. SW
Room 2945 South Building
Washington, DC 20250-0268
Direct: 202-821-9683
lisa.brines@ams.usda.gov

Dear Dr. Brines

Attached find our new
Petition of substance for inclusion on the National List of Substances allowed in Organic Production and Handling

Indole-3-butyric acid, IBA
To be allowed in 'Organic Production' for purposes of plant propagation from cuttings, for use on annual, perennial and woody plants. Application shall be made in enclosed structures. Rates shall be limited to aqueous solution up to 2500 ppm IBA and dry powder up to 0.8% IBA.

Please reference our prior NOP petition for IBA, The NOP Technical Report, and NSOP recommendations.

Regards

Joel Kroin
President

Officer:	Dr. Lisa Brines
Agency:	National List Manager, Standards Division
	USDA/AMS/NOP, Standards Division
Address:	Attention: Stacy Jones King
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	Room 2945 South Building
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PETITIONER:

Company:	Hortus USA Corp.
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	New York NY 10113
Email address:	support@hortus.com
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Fax:	(212) 624-0202
Contact person:	Joel Kroin
Title:	President

DATE: August 8, 2012

RE: Agricultural Marketing Service, 7 CFR Part 205, [Docket No. MS-TM-06-0223;TM-06-12] National Organic Program Submission of Petitions of Substances for Inclusion on the National List of Substances Allowed and Prohibited.

PETITION:
Petition of substance for inclusion on the National List of Substances allowed in Organic Production and Handling

Indole-3-butyric acid, IBA
To be allowed in 'Organic Production' for purposes of plant propagation from cuttings.
For use on annual, perennial and woody plants.
Application shall be made in enclosed structures.
Rates shall be limited to aqueous solution up to 2500 ppm IBA and dry powder up to 0.8% IBA.

INTRODUCTION

Indole-3-butyric acid (IBA) is a natural substance found in plants. It is used to propagate new plants from cuttings, the most used substance for this purpose. It is effective, and safe to the environment, humans and animals. For commercial use, IBA must be synthesized.

A. PETITION

IBA is to be allowed in 'Organic Production'

- Use is limited to plant propagation from cuttings,
- All plant types which can be propagated from cuttings can be used: annual, perennial and woody plants.
- Rates are to be limited in aqueous solution up to 2500 ppm IBA and dry powder up to 0.8% IBA.
- 'Spot use' application is to be made in 'enclosed structures'. Field broadcasting is not allowed.
- Solutions are specified to be 'aqueous' assuring only water can be used as the solute.

B. USES

IBA is used, once or few times, at extremely low rates near the time cuttings are taken. It is many weeks or months after application before crops are taken.

C. NEED FOR IBA BY ORGANIC FARMERS

Plants propagated from cuttings are clonal copies of the mother plant. IBA allows the plant cuttings to produce new roots rapidly. IBA is used to propagate most plants which can be reproduced from cuttings. IBA is used these purposes:

1. Many crops are impossible to root from cuttings, such as bay and rosemary.

2. Crops which are difficult to root, and take a long time to develop roots are under stress susceptible to pathogenic fungi such as Phytophthora. Rapid root formation eliminates stress and allow plants to overcome these fungi.

3. Many seedless crop plants are not available to the organic market such as seedless tomato, cucumber, melon, and squash. Propagation from cuttings using IBA allows growers to produce many kinds of seedless crops like beans, tomato, cucumber, squash, and melon. Propagation from cuttings using IBA yields new plants that are identical to the mother plant..

4. Many crops can only retain flavor if clonal propagated from cuttings such as mints and basal. Seed propagated basal plant variable flavors; propagation by cuttings using IBA produce exact flavors as the mother plants

5. Organic crops that are currently propagated using IBA or TC must be kept one year under organic conditions before being called ‘organic’. IBA on the approved list allows crops to be taken any time after propagation. For example, strawberry and raspberry are sometimes propagated by tissue culture or from cutting using IBA. During the first year of growing under organic conditions they are considered conventional. Propagation using allowed IBA will allow growing under ‘organic’ conditions to be called ‘organic’ any time.

D. LONG HISTORY OF SAFETY

As a natural substance, IBA has been shown to have no detrimental effect on the environment. Since identification in 1934, IBA has been shown to be safe to humans and the environment.

E. ORGANIC PRODUCTION STATUS

There are presently no allowed substances with root inducing ability on the NOP National List. Presently, if IBA is used on cuttings, growers must grow plants one year under organic conditions before the crop can be certified ‘organic’.

F. QUALITY CONTROL

IBA is required to registered with the US EPA for use in plant growing. The US EPA registration requires that all ingredients must disclosed in the Confidential Statement of Formula if over 0.1%. ‘Manufacturing Use’ and ‘End Use’ products have certified disclosed content.

G. GROWER COMMENTS

Jim Walsh
Greenhouse Manager
Nourse Farms
41 River Road
S Deerfield MA 01373
413-665-2658

Norse Farms is a primary plant supplier of blackberry, blueberry, currant, elderberry, gooseberry, raspberry, and strawberry. Nourse Farms uses IBA solutions to propagate plants from cuttings. They tell their organic grower customers to keep their plants one year under organic conditions before calling the crop 'organic'.

Aris Horticulture, Green Leaf Plants division
PO Box 230
Barberton OH 44203
330-745-2l43

Aris Horticulture, formerly Yoder Brothers, is one of the largest US suppliers of rooted and un-rooted perennial plant cuttings. Their herb selection includes lavender, metha (mint), slavia (sage), thymus (thyme) , stevia, oregano, rosemary, basal and catnip. Green Leaf uses IBA solutions to propagate plants from cuttings. They sell their rooted cuttings to growers who can take their crop within several weeks after growing our. Green Leaf would like to have IBA on the allowed List since their customers would have a selection of organically produced cuttings which can be sold as organic crops.

Cliff Hoogland
General Manager
Phytotronics Inc
13688 Rider Trail North
Earth City MO 63045
314-770-0717

Phytotronics is a primary supplier of materials used by plant growers. They say that they have frequent requests from organic growers for plant rooting products which can be used in organic production. Currently Phytotronics has IBA which is not on the approved List. Phytotronics has a product demand from organic growers which they can not satisfy.

Andy McNitt
McNitt Growers
78 Oak Ridge Land
Carbondale IL 62909
618-687-3563

McNitt Growers is a primary supplier of strawberry plants used in commercial production. They use IBA in their production. The are unable to economically keep their plants for two growing seasons as organic, to be sold as organic to organic growers. They sell their plants after the first growing season.

Mark Langan
Owner
Mulberry Creek Herb Farm
3312 Bogart Road
Huron, Ohio 44839
419- 433-6126

Mulberry Creek Herb Farm is a Certified organic grower. They are unable to produce production crops of difficult to root rosemary and lavender. They have very low percentage of rooting of cuttings. They specifically said they require IBA for use in their plant propagation. They also say they are unable to purchase certified organic rooted cuttings.

Item A	**Section the petitioned substance will be included on the National List.**	
	Synthetic substances allowed for use in organic crop production,§ 205.601.	
Item B		
1.	**Petition**	
	The substance's chemical or material common name.	Indole-3-butyric acid
	Indole-3-butyric acid, IBA CAS number 133-32-4 Empirical formula C12 H13 NO2 To be allowed in 'Organic Production' for purposes of plant propagation from cuttings. For use on annual, perennial and woody plants. Application shall be made in enclosed structures. Rates shall be limited to aqueous solution up to 2500 ppm IBA and dry powder up to 0.8% IBA.	
2.	**The manufacturer's or producer's name, address and telephone number and other contact information of the manufacturer/producer of the substance listed in the petition**.	
	Hortus USA Corp. PO Box 1956 Old Chelsea Station New York NY 10113 support@hortus.com Phone: (212) 929-0927 FAX (212) 624-0202 Contact: Joel Kroin, President	
3.	**The intended or current use of the substance such as use as a pesticide, animal feed additive, processing aid, non-agricultural ingredient, sanitizer or disinfectant.**	
	Propagate new plants from cuttings by inducing root formation.	

4. First part	**A list of the crops for which the substance will be used. If used for crops or livestock, the substance's rate and method of application must be described.**	
	IBA IS USED ON MOST ANNUAL, PERENNIAL AND WOODY PLANTS WHICH CAN BE ROOTED (PROPAGATED) FROM CUTTINGS Among uses, IBA is used to propagate seedless plants such as tomato and cucumber. Annuals: such as tomato, basal, squash, melon, cucumber, cotton (for oil) Perennial plants: such as strawberry, blueberry, raspberry, lavender, rosemary, thyme, mint, oregano, salvia, sage Woody plants: such as grape, apple, rose (for rose hips)	
	IBA IS USED BY TWO PRODUCT TYPES (1) DRY POWDERS AND (2) SOLUTIONS	
	(1) DRY POWDERS WHICH CONSIST OF IBA MIXED INTO TALC. A. BASAL DRY DIP METHOD Rates: 0.1% IBA up to 0.8% IBA The basal end of the cuttings are dipped approximately one inch in to the dry powder. The cuttings are then stuck into media. After, cuttings are maintained under appropriate environmental control until roots are formed.	
	(2) SOLUTIONS WHICH CONSIST OF IBA DISSOLVED INTO WATER (AQUEOUS SOLUTION). The solutions are used in one of five ways.	

	A. FOLIAR TOTAL IMMERSE METHOD Rates: up to 1500 ppm IBA The basal end of the cuttings are dipped approximately one inch in to the solution. The cuttings are then stuck into media. After, cuttings are maintained under appropriate environmental control until roots are formed	
	B. FOLIAR SPRAY DRIP DOWN METHOD Rates: up to 1500 ppm IBA The cuttings are stuck into media. The solution is spot sprayed onto the leaves until droplets form. After, cuttings are maintained under appropriate environmental control until roots are formed.	
	C. BASAL QUICK DIP METHOD Rates: up to 2500 ppm IBA The basal end of the cuttings are dipped approximately one inch in to the solution about five seconds. The cuttings are then stuck into media. After, cuttings are maintained under appropriate environmental control until roots are formed	
	D. BASAL LONG SOAK METHOD Rates: up to 300 ppm IBA The basal end of the cuttings are dipped approximately one inch in to the solution, about 12-24 hours. The cuttings are then stuck into media. After, cuttings are maintained under appropriate environmental control until roots are formed.	
	E. IF CUTTINGS ARE SLOW TO ROOT Foliar Spray Drip Down Method can be used at weekly applications until roots form. FOLIAR SPRAY DRIP DOWN METHOD (see above) Rates: up to 1500 ppm IBA	
4. Second part	**If used for handling (including processing), the substance's mode of action must be described.**	

	REFERENCE ATTACHED FILES: "REFERENCES-IBA root formation physiology" "REFERENCES-IBA efficacy" Many references IBA induces plant cells to form new roots by cell division even when other natural substances are unable to do so. Scientists have not been able to fully determine the mode of action.	
	REFERENCE: June 22, 2011, Technical Evaluation Report, Compiled by the Technical Services Branch for the USDA National Organic Program SEE ANSWER TO: Characterization of the petitioned substance "Action of the substance" "Evaluation question #11": Plant hormones [including IBA] regulate the cell division and elongation in general, but each group of plant hormones further posses some dedicated functions. For example .. stimulate root growth" "Even though the physiological roles of plant hormones [including IBA] are not well understood, plant hormones, as a whole category of substances, are considered essential and practically used in various plant propagation and growth applications."	
5.	**A. The source of the substance and a detailed description of its manufacturing or processing procedures from the basic component(s) to the final product.**	
	ATTACHED FILES: "REFERENCES-IBA mfg method patents MSDS"	
	B. Natural source and availability to organic growers	

	ATTACHED FILES: "REFERENCES -IBA natural" IBA is a natural substance produced by plants. There are no commercial sources for naturally produced IBA. All commercial sources of IBA must be produced by synthesis.	
	C. Manufacturing method (synthesis)	
	ATTACHED FILES: "REFERENCES-IBA mfg method patents MSDS"	
6.	**A summary of any available previous reviews by State or private certification programs or other organizations of the petitioned substance.**	
	NOP IBA Petition by Hortus USA "petitioned substance will be included on the National List." Petition dated August 29 2008	
	Petition NOB Technical report dated June 22, 2011	
	Petition NSOB formal recommendation December 2, 2011	
7.	**Information regarding EPA, and State regulatory authority registrations, including registration numbers.**	Below
	Numbers of products: 18 producing companies, 31 US EPA registered IBA products labeled for plant propagation from cuttings "REFERENCES-USEPA documents" IBA containing products 08 2012.pdf Source: http ://ppis.ceris.purdue.edu	

	IBA is required to be registered for use in plant growing under FIFRA Regulatory status: ATTACHED FILES: "REFERENCES-USEPA documents" USEPA FIFRA definitions and registration requirement.pdf USEPA IBA fact sheet 1992.pdf USEPA IBA RED 1992.pdf USEPA IBA status 4-12-2012.pdf	
	Typical US EPA products which are registered for plant propagation from cuttings Hortus IBA Water Soluble Salts US EPA registration number 63310-22 Rhizopon AA #1 (0.1) US EPA registration number 63310-19 Rhizopon AA #2 (0.3) US EPA registration number 63310-20 Rhizopon AA #3 (0.8) US EPA registration number 63310-21 Rhizopon AA Water Soluble Tablets US EPA registration number 63310-8	
	ATTACHED FILES: "REFERENCES-IBA Labels and MSDS" Hortus IBA Water Soluble Salts Approved Label consumer_commercial 10 18 2010.pdf Rhizopon AA1 Approved Label consumer_commercial 10 18 2010.pdf Rhizopon AA2 Approved Label consumer_commercial 10 18 2010.pdf Rhizopon AA3 Approved Label consumer_commercial 10 18 2010.pdf Rhizopon AA Water Soluble Tablets Approved Label consumer_commercial 10 18 2010.pdf	
8.	**The Chemical Abstract Service (CAS) number or other product numbers of the substance**	

	Indole-3-butyric acid IBA CAS number 133-32-4 Empirical formula C12 H13 NO2	
	Labels of products that contains the petitioned substance.	
	ATTACHED FILES: "REFERENCES-IBA Labels and MSDS" Hortus IBA Water Soluble Salts Approved Label consumer_commercial 10 18 2010.pdf Rhizopon AA1 Approved Label consumer_commercial 10 18 2010.pdf Rhizopon AA2 Approved Label consumer_commercial 10 18 2010.pdf Rhizopon AA3 Approved Label consumer_commercial 10 18 2010.pdf Rhizopon AA Water Soluble Tablets Approved Label consumer_commercial 10 18 2010.pdf	
9.	**The substance's physical properties and chemical mode of action including:** **a) Chemical interactions with other substances, especially substances used in organic production** **b) toxicity and environmental persistence;** **c) environmental impacts from its use and/or manufacture** **(d) effects on human health** **e) effects on soil organisms, crops, or livestock**	

	REFERENCE: June 22, 2011, Technical Evaluation Report, Compiled by the Technical Services Branch for the USDA National Organic Program SEE ANSWER TO: “Characteristics of petitioned substances” CHARACTERIZATION OF PETITIONED SUBSTANCE Composition of the Substance: Indole-3-butyric acid (IBA) is a white to tan powder or crystalline solid with a slight characteristic odor. Formula: C12H13NO2. Formula weight: 203.24. IBA may be viewed as a chemical compound with these two composing units: indole and butyric acid. A similar compound is indole-3-acetic acid (IAA: CAS Number: 87-51-4. C10H9NO2. Formula weight: 21 175.19). I AA may be viewed as a compound with these two composing units: indole and acetic acid.
	PROPERTIES OF THE SUBSTANCE: IBA is stable at 2-8°C and should be stored in a cool place. The melting point of IBA is 121-125°C. IBA does not contain combustible liquids but decomposes to toxic fumes, such as NOx, carbon monoxide, and carbon dioxide in fire. Its density is 0.60 g cm-3 at ambient temperature, and pH 3.54 for a 1% solution by weight dispersion in water. IBA is practically insoluble in chloroform, but is soluble in alcohol, ether and acetone. The solubility of IBA in water is 250 mg L-1 (EPA, 2010). In order to make an aqueous IBA solution for purposes such as applying IBA to plant roots, IBA was dissolved with methanol, and this methanol solution was further diluted with water to make an aqueous IBA solution. The salt form of IBA, such as the sodium salt of indole-3-buryrate, is soluble in water. IBA is made into water-soluble and water-insoluble products. IBA decomposes when exposed to light (e.g. Nor Aini et al., 2009). However, specific information such as how fast IBA decomposes under various ambient conditions is still limited.

	REFERENCE: June 22, 2011, Technical Evaluation Report, Compiled by the Technical Services Branch for the USDA National Organic Program SEE ANSWER TO: "Evaluation Question #9: Discuss and summarize findings on whether the petitioned substance may be 275 harmful to the environment." As given in Question #5, the used amount of IBA in various applications is trace to minor. EPA (1992) listed a set of facts and reasonable assumptions about IBA's effect on organisms and environment: the applied amount of IBA is low; IBA is a plant hormone but not a toxicant or repellant; IBA is structurally and functionally similar to other natural auxins; and IBA might also occur naturally in plants. "IBA has been shown to be practically nontoxic to avian species. … IBA should not cause any adverse effects to avian wildlife,". IBA is not known to be phytotoxic. IBA should not cause any adverse effects to mammalian wildlife. EPA (2010) further discussed the toxicity of IBA to nontarget insects and to threatened species, and indicated that testing data may not necessarily be needed urgently. IBA is shown to be slightly toxic to fish and aquatic invertebrates. No data or evidences are listed in the PAN database about the harmful effect of IBA to the environment, except some slight toxicity towards fish.	
	CONCLUSIONS	
	(1) potential of such substance for detrimental chemical interactions with other materials used in organic farming systems	

	REFERENCE: June 22, 2011, Technical Evaluation Report, Compiled by the Technical Services Branch for the USDA National Organic Program SEE ANSWER TO: "Evaluation Question #7:" Describe any known chemical interactions between the petitioned substance 243 and other substances used in organic crop or livestock production or handling. Describe any environmental or human health effects from these chemical interactions. IBA potentially reacts with strong oxidizers IBA is used and found effective as growth promoter and rooting stimulator in various applications. The amount used for this purpose is from trace to minor, as given in Question #5. Plant hormones are not nutrients to plants but chemicals, even at trace to minor amounts, regulate plant growth. The stimulating effect of IBA is synergetic with other chemicals and bacteria. Auxins such as IAA and IBA stimulated rooting in the first and the second phases of rooting but suppressed the rooting in the third phase. Therefore, an excessive usage of IBA might lead to some unfavorable consequences in plant growth. However, the literature about these potential unfavorable consequences is limited. Dipping cuttings in IBA solution or powder and foliar spray of IBA are the primary means of applying IBA for plant growth and plant propagation. Foliar spray of IBA is used in enhancing rooting and crop yield. "Applicability to a wide range of crops has yet to be established. Examination of the variability in absorption and translocation of foliar-applied auxin to the site of root initiation may merit further study," . The used amount is limited, as given in Question #5. The petition claimed "no interactions" of IBA with other substances used in organic production. The literature about IBA's potentially detrimental chemical interaction with other substances used in organic crop or livestock production is scarce.	
	(2) toxicity and mode of action of the substance and of its breakdown products or other contaminants and their persistence and areas of concentration in the environment	

	REFERENCE: June 22, 2011, Technical Evaluation Report, Compiled by the Technical Services Branch for the USDA National Organic Program SEE ANSWER TO: "Evaluation Question #7:" No data or evidence is listed in the PAN database about "terrestrial ecotoxicity". Slight toxicity is listed towards fish in the category of "aquatic ecotoxicity" .	
	(3) probability of environmental contamination during manufacture, use, misuse or disposal of such substances	
	MANUFACTURE: Produced in small production lots under laboratory control DISPOSAL: None when disposed using approved label instructions.	
	Use: REFERENCE: June 22, 2011, Technical Evaluation Report, Compiled by the Technical Services Branch for the USDA National Organic Program SEE ANSWER TO: Category 1, item 3: No evidence of harm. SEE ANSWER TO: Category 1, item 6: Are there adverse biological and chemical interations in agro-ecosystem. This petition specially requires application in an 'enclosed structure', thereby eliminating concern for area broadcast risk.	

	REFERENCE: "REFERENCES-USEPA documents" USEPA IBA fact sheet 1992.pdf IV. Assessing Risks to the Environment No risks to the environment are expected from use of this active ingredient because 1) it does not harm animals or plants in the tiny amounts used, 2) it acts as a plant growth enhancer, 3) it does not persist in the environment, 4) it is closely related to naturally occurring substances.	
	(4) effect of the substance on human health.	
	None when applied using approved personal protection equipment (PPE). REFERENCE: "REFERENCES-USEPA documents" USEPA IBA fact sheet 1992.pdf III. Assessing Risks to Human Health With the exception of certain workers, no harm is expected from use of indole-3-butyric acid. The active ingredient is not toxic to humans or other mammals. Furthermore, indole-3-butyric acid is effective at very low concentrations--often several orders of magnitude below 1%. It is applied at very low rates compared with most other pesticides. In animals, indole-3-butyric acid is rapidly broken down to a closely related, harmless chemical that occurs naturally in living organisms. Eye irritation to certain workers is EPA' s only health concern for products containing indole-3-butyric acid. For products that may cause eye irritation, workers (such as mixers and applicators) are required to use protective eyewear, such as goggles, face shield, or safety glasses.	
	(5) effects of the substance on biological and chemical interactions in the agroecosystem	

	None. The US EPA has stipulated: “it does not persist in the environment”. REFERENCE: June 22, 2011, Technical Evaluation Report, Compiled by the Technical Services Branch for the USDA National Organic Program SEE ANSWER TO: “Evaluation question #7" EPA stipulated that IBA does not persist in the environment . Evaluation Question #5: Describe the toxicity, mode of action and breakdown products of the petitioned substance any known toxic or other adverse action of the substance and/or its breakdown 204 products. Relevant to Questions #5 - #10, the common amount used in various applications was from 0.5 mg L-1 to less than 1% (10,000 mg L-1) in solution. “All currently registered end products formulated with IBA are applied in ultra-low quantities, up to 7 mg active ingredient/acre/crop season for the crop uses, and similar low applicator exposure for ornamental plant propagation,” The primary application is to dip plant cuttings in IBA solution or IBA powder for inducing the adventitious root formation. Foliar spray of IBA is used in enhancing crop yield. IBA is being petitioned to be used as a plant growth promoter. “Low toxicity” was claimed by the petitioner for both the active ingredient (i.e. IBA) and possible breakdown products. EPA evaluated the toxicity of IBA based on the following rational. IBA is similar in structure and function to naturally occurring IAA (actually, IBA, based on the recent literature, is also naturally occurring). IBA is metabolized to IAA in the human body. IBA has a non-toxic mode of action. EPA concluded that IBA has low acute toxicity with the exception that IBA is an eye irritant. 40 CFR 180.1158 exempted IBA from the requirement of a tolerance for residues of IBA in or on food commodities when used as plant regulators. “All generic toxicology data requirements have been waved 221 for IBA,” EPA (1992). EPA stipulated that IBA does not persist in the environment (EPA, 1992; EPA, 2010). Technical Evaluation Report Indole-3-butyric acid (IBA) Crop Production June 22, 2011 Page 6 of 17	
	(6) alternatives to using the substance in terms of practices or other materials available	

	AS DESCRIBED BELOW: No alternative substances are available as US EPA registered plant growth regulator substances. All plant growth regulators are required to be registered with the US EPA under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). IAA, like IBA, has no natural sources for commercial use by plant growers. REFERENCE: June 22, 2011, Technical Evaluation Report, Compiled by the Technical Services Branch for the USDA National Organic Program SEE ANSWER TO: "All natural substances used in place of IBA" Other auxin compounds in general are active only at higher concentrations than IAA and their role in growth remains largely unknown. In recent years, some of these other auxins such as IBA were found more effective in stimulating rooting than IAA during certain developmental stages or in certain plant species . IBA was used in recent researches for promoting root growth.
10.	**Safety information about the substance including a Material Safety Data Sheet (MSDS) and a substance report from the National Institute of Environmental Health Studies.**
	ATTACHED FILES: "REFERENCES-IBA Labels and MSDS" MSDS Hortus IBA Water Soluble Salts.pdf MSDS Rhizopon AA1.pdf MSDS Rhizopon AA2.pdf MSDS Rhizopon AA3.pdf MSDS Rhizopon AA Water Soluble Tablets.pdf MSDS IBA RESEARCH.pdf

	REFERENCE: "REFERENCES-USEPA documents" "USEPA IBA RED 1992.pdf " B. Human Health Assessment 1. Toxicology Data The Agency has waived all data requirements on the active ingredient because of the expected, extremely low exposures to those involved in the use of products containing IBA and due to the negligible dietary exposures expected from the use of IBA on food and feed crops. IBA is exempt from tolerances of residues on crops (40 CFR 180.1099). All registered products are formulated with IBA in low percentages of IBA, from 0.0004 to 4.5, and are applied in ultra-low quantities, up to 7 mg active ingredient/acre/crop season, for the crop uses. Use of products for the ornamental plant propagation use also results in low applicator exposure to IBA. Additionally, these products have low acute toxicity as suggested from data of at least one formulated product (Toxicity Categories ill and IV, no dermal irritation, and moderate eye irritation, perhaps from another active or an inert ingredient in the product formulation. 2. Occupational and Residential Exposure There is potential for occupational exposure to IBA during dipping and transplanting activities and mixing, loading and spraying activities. However, since the Agency does not have concerns about any toxicological endpoints, the Agency has not required exposure data. The Agency has no significant exposure concerns other than appropriate label precautions for eye protection for mixers, loaders, and applicators.

11.	**Research information about the substance which includes comprehensive substance research reviews and research bibliographies, including reviews and bibliographies which present contrasting positions to those presented by the petitioner in supporting the substance's inclusion on or removal from the National List.** **For petitions to include non-organic agricultural substances onto the National List, this information item should include research concerning why the substance should be permitted in the production or handling of an organic product.** **Availability of organic alternatives.** **Commercial availability does not depend upon geographic location or local market conditions**	
	REFERENCE “REFERENCES-IBA efficacy” Plant propagation from cuttings produces clonal copies of the mother plant. IBA is the most use substance for this purpose	

	No useful substances are available to organic growers REFERENCE: June 22, 2011, Technical Evaluation Report, Compiled by the Technical Services Branch for the USDA National Organic Program SEE ANSWER TO: Evaluation question #11 Currently, "European and North American regulations do not allow the use of synthetic products to obtain organic vegetative propagation materials," Centeno and Gomez-del-Campo (2008). Centeno and Gomez-del-Campo (2008) briefly introduced methods in plant propagation without using synthetic materials. Cuttings were enclosed with germinating seeds to stimulate root formation since germinating seeds contained natural auxins. Fungi produced auxins and contained proteins, carbohydrates, lipids, minerals, and vitamins. Algae also contained IAA, proteins, lipids and carbohydrates. Yeast extract was used for root growth in Glehnia littoralis. Mineral nutrients and vitamins were found improving the rooting of cuttings (Christov and Koleva, 1995). Specifically, Centeno and Gomez-del-Campo (2008) confirmed that IBA was effective in promoting rooting	
	IBA is commercial availability worldwide. REFERENCE: REFERENCES-USEPA documents IBA containing products 08 2012.pdf	
12.	**A "Petition Justification Statement" which provides justification for any of the following actions requested in the petition: A. Inclusion of a Synthetic on the National List, §§205.601, 205.603,205.605(b)**	

	• Explain why the synthetic substance is necessary for the production or handling of an organic product. **• Describe any non-synthetic substances, synthetic substances on methods that could be used in place of the petitioned synthetic substance.** **• Describe the beneficial effects to the environment** **• Describe the beneficial effects to the human health**	
	SEE: "INTRODUCTION" at the beginning of this petition	
	Indole-3-butyric acid (IBA) is a natural substance found in plants. It is used to propagate new plants from cuttings, the most used substance for this purpose. It is effective, and safe to the environment, humans and animals. For commercial use, IBA must be synthesized.	
	PETITION USE AND RATES IBA is to be allowed in 'Organic Production' Use is limited to plant propagation from cuttings, All plant types which can be propagated from cuttings can be used: annual, perennial and woody plants. Rates are to be limited in aqueous solution up to 2500 ppm IBA and dry powder up to 0.8% IBA. 'Spot use' application is to be made in 'enclosed structures'. Field broadcasting is not allowed. Solutions are specified to be 'aqueous' assuring only water can be used as the solute.	
	USES IBA is used, once or few times, at extremely low rates near the time cuttings are taken. It is many weeks or months after application before crops are taken.	

NEED FOR IBA BY ORGANIC FARMERS

Plants propagated from cuttings are clonal copies of the mother plant. IBA allows the plant cuttings to produce new roots rapidly. IBA is used to propagate most plants which can be reproduced from cuttings. IBA is used these purposes:

Many crops are impossible to root from cuttings, such as bay and rosemary.

Crops which are difficult to root, and take a long time to develop roots are under stress susceptible to pathogenic fungi such as Phytophthora. Rapid root formation eliminates stress and allow plants to overcome these fungi.

Many seedless crop plants are not available to the organic market such as seedless tomato, cucumber, melon, and squash. Propagation from cuttings using IBA allows growers to produce many kinds of seedless crops like beans, tomato, cucumber, squash, and melon. Propagation from cuttings using IBA yields new plants that are identical to the mother plant..

Many crops can only retain flavor if clonal propagated from cuttings such as mints and basal. Seed propagated basal plant variable flavors; propagation by cuttings using IBA produce exact flavors as the mother plants

Organic crops that are currently propagated using IBA or TC must be kept one year under organic conditions before being called ‘organic’. IBA on the approved list allows crops to be taken any time after propagation. For example, strawberry and raspberry are sometimes propagated by tissue culture or from cutting using IBA. During the first year of growing under organic conditions they are considered conventional. Propagation using allowed IBA will allow growing under ‘organic’ conditions to be called ‘organic’ any time.

LONG HISTORY OF SAFETY

As a natural substance, IBA has been shown to have no detrimental effect on the environment. Since identification in 1934, IBA has been shown to be safe to humans and the environment

ORGANIC PRODUCTION STATUS

There are presently no allowed substances with root inducing ability on the NOP National List. Presently, if IBA is used on cuttings, growers must grow plants one year under organic conditions before the crop can be certified ‘organic’.

QUALITY CONTROL

IBA is required to registered with the US EPA for use in plant growing. The US EPA registration requires that all ingredients must disclosed in the Confidential Statement of Formula if over 0.1%. ‘Manufacturing Use’ and ‘End Use’ products have certified disclosed content.

	REFERENCE: June 22, 2011, Technical Evaluation Report, Compiled by the Technical Services Branch for the USDA National Organic Program SEE ANSWER TO: "EVALUATION QUESTION #12 As being contrasted to the overall pictures of plant propagation and plant hormones given above, IBA is one of numerous plant hormones. Propagation from cuttings is one of numerous plant propagation processes. Successful rooting from stem cuttings depends on numerous factors such as stock plant management, timing, types of cuttings, rooting environment (light, temperature, moisture, etc), and ten other or so factors. Applying plant hormones is one of these factors. In addition to IBA, NAA is commonly used in conventional operations; however NAA is prohibited under the National Organic Program standards. The application of plant hormones is not a method for all scenarios since this application is further limited by numerous factors: amount, timing, type-mismatch, solution or solid, etc.

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MSDS IBA Technical

MSDS Rhizopon AA #1, US EPA reg# 63310-19

MSDS Rhizopon AA #2, US EPA reg# 63310-20

MSDS Rhizopon AA #3, US EPA reg# 63310-21

MSDS Rhizopon AA Water Soluble Tablets, US EPA reg# 63310-8

MSDS Hortus IBA Water Soluble Salts, US EPA reg #63310-22

US Epa Stamped Approval Labels:

Rhizopon AA #1, US EPA reg# 63310-19

Rhizopon AA #2, US EPA reg# 63310-20

Rhizopon AA #3, US EPA reg# 63310-21

Rhizopon AA Water Soluble Tablets, US EPA reg# 63310-8

Hortus IBA Water Soluble Salts, US EPA reg# 63310-22

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USEPA Registered IBA containing products, labeled for plant propagation from cuttings

USEPA CSF Form 8560-3

USEPA Definition of Tolerance

USEPA FIFRA Definitions

USEPA IBA Exempt from Tolerance

USEPA IBA Fact Sheet 1992

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Gas Chromatographic Analysis of Acidic Indole Auxins in Nicotiana[1]

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Received October 21, 1968.

Abstract. **Acidic indole auxins have been extracted from *N. glauca, N. langsdorffii* and their 2 tumor-prone 4n- and 2n-hybrids. After purification of the extracts and thin-layer chromatography, acidic indoles were subjected to esterification and gas chromatography. The esters of 4 indole acids were detected and determined: indole-3-acetic acid, indole-3-carboxylic acid, indole-3-propionic acid and indole-3-butyric acid. The indolic nature of fractionated samples was confirmed by spectrophotofluorometry and the physiological significance of the indole esters proven in a biotest. A substantial increase in extractable indole-3-butyric acid in the tumor-prone hybrids suggests an additional pathway of auxin synthesis in these tissues.**

An indole auxin, presumably indole-acetic acid (IAA), was described as the main growth hormone in several *Nicotiana* species, their hybrids and hybrid derivatives (2, 3, 4). Identification of IAA was based on ether extraction and separation by paper- and thin-layer chromatography, coupled with an *Avena* biotest. A quantitative comparison of the extracted acidic indole compound revealed a higher content in the tumor-prone genotypes of *Nicotiana*, as compared to their non-tumor forming parent species. However, obtaining more detailed information about other naturally occurring indole auxins in the plant extracts was made difficult by the large number of these substances and the minute concentrations in which they usually occur.

Because of similarities in molecular structure these indoles overlap on chromatograms or possess similar R_F values in the same partition solvent systems (24, 30). Therefore, a highly sensitive method is necessary for a more detailed identification of indole-auxins at physiological concentrations. Gas-liquid chromatography and spectrophotofluorometry have proven to be valuable for investigations of synthetic indoles (6, 9, 15, 21, 27). The present communication describes the application of these analytical procedures to biological material. Specifically, extracts of *Nicotiana glauca, N. langsdorffii* and their tumor-prone 4n- and 2n-hybrids have been examined for the occurrence of different acidic indole auxins. The most striking aspect observed was an unusually high level of indole-butyric acid in tumor-prone hybrids.

[1] This work was supported by USPHS Grants CA-04890, CA-06927 and FR-05539 from the National Cancer Institute and by an appropriation from the Commonwealth of Pennsylvania.

Materials and Methods

Plant Material. Plants of *N. glauca, N. langsdorffii* and their 2 tumor-prone hybrids, the amphidiploid hybrid GGLL (4n) and the F_1-hybrid GL (2n) were grown in the greenhouse for 4 to 5 months. Before the onset of flowering and tumor-formation, the top 10 to 15 cm stems of the plants were excised, the leaves removed and the pieces cut and frozen immediately. For each extraction 30 g of tissue (fr wt) from 4 to 5 plants was used.

Preparation of Samples for Gas Chromatography. In order to investigate indole containing samples in the physiological range of concentrations by gas chromatography, a thorough and selective purification of tissue extracts is essential to eliminate many interfering substances (27). The sequence of steps was as follows: A) fractionation of plant extracts into major indole groups; B) thin-layer chromatography of the acidic indole group on silica gel; C) methylation of acidic indoles; D) gas chromatography; E) spectrophotofluorometry; F) biotest.

Frozen tissue samples were extracted for 20 hr at 3° with cold, peroxide-free ether at pH 3.5. The ether was evaporated and the extract taken to dryness under reduced pressure. The fractionation scheme described in detail by Powell (21) was used to obtain the acidic indole fractions of the various *Nicotiana* extracts. Any remaining non-indolic compounds like phenols and phenol derivatives were removed by partitioning the acidic indole-fraction with 2% $NaHCO_3$. The residue of the aqueous indole-fraction was taken up in a known volume of purified methanol and streaked on thin-layer chromatographic sheets (Eastman Chromagram Sheet, type K301R, Silicagel). Chromatograms were run in *n*-butanol-water-ammonia (10:10:1, upper phase). Four to 5 μg of synthetic indole-3-acetic acid (IAA)

were run as controls and the R_F of IAA determined by the method of Gordon and Weber (14) with ferric chloride-perchloric acid. The dried chromatograms, 8 cm in length, were divided into 10 equal transverse strips and the R_F sections corresponding to the location of synthetic IAA in controls and known to contain natural acidic indole derivatives, were scraped off and transferred to a millipore filter syringe. The auxin containing silica gel was eluted twice each with 1 ml of methylene chloride and 1 ml of 10% methyl alcohol. The combined eluates were then subjected to esterification, using diazomethylation (20, 22) and a micromethod described by Powell (21) for the methylation of small indole samples.

As references for naturally occurring indole acids, the following synthetic indole acids which are commercially available, were esterified by the same methods: indole-acetic acid (IAA), indole-carboxylic acid (ICA), indole-propionic acid (IPA), indole-butyric acid (IBA), indole-lactic acid (ILA), indole-acrylic acid (IAcA) and 5-hydroxyindole-acetic acid (5-OH-IAA). Although the physiological significance of indole-pyruvic acid as an intermediate product of IAA synthesis from tryptophan is recognized, this indole compound could not be included in the present investigation because of its rapid decomposition in the alkaline solvent system (*n*-butanol-ammonia-water) used for thin-layer chromatography (1, 23, 28). Ethyl indole-3-acetate (IAA-EE), obtained from commercial sources, was selected as a standard since it has an intermediate retention volume and is useful over a wide range of column temperatures (27).

Gas Chromatography. The gas chromatograph used was a Glowall Model 310 connected to a Photovolt Chart Recorder. It was equipped with an interchangeable hydrogen flame detector and run at a 300 volt DC current. The coiled columns were 6 feet long and had an inner diameter of 6 mm. They were packed either with 3% SE-30 (methyl vinyl silicone rubber, General Electric Company) or 3% silicone QF-1 (a Dow-Corning Corporation Fluorinated Silicone). The column temperature was kept at 190°, the vaporizing block temperature at 235° and the detector oven at 255°. Argon was used as the carrier gas at a flow rate of 30 ml/min at 20 to 22 lbs/in^2 pressure. Two to 5 μg samples of esterified indoles dissolved in acetone were injected into the vaporizing block.

In an attempt to analyze fractions of the gas chromatographed samples by spectrophotofluorometry and bioassay, the hydrogen flame detector was replaced by an argon ionizing detector (run on 1000 DC volts; radium source: 22.5 μcuries) which allows collection of fractionated samples after passing through the detector chamber. Small bore teflon tubing was connected to the detector outlet and led through an ice filled Dewer container. Condensation of the indole esters occurred in the cooled teflon tubing which was consequently rinsed with acetone. For the spectrophotofluorometric measurements the acetone was evaporated and the residue dissolved in 1 ml of ethyl alcohol and transferred to a quartz cuvette. Readings were made at an activation wavelength of 280 mμ and a fluorescence wavelength of 360 mμ. These wavelengths are near maxima for most indoles (except for 5-OH indoles which are activated at 295 mμ).

For bioassay the condensed residues from the teflon tubing were rinsed with ethanol water, transferred to agar blocks (2.5 × 2.5 × 1.3 mm) and subjected to an *Avena* curvature test (2, 4, 26).

Results

A) *Gas Chromatography.* Diazomethylation proved to be a successful method for esterification of naturally occurring indole acids. In recovery experiments with samples of synthetic indoles esterification was nearly complete, *i.e.* 90 to 96% was recovered as methyl ester. These data are in agreement with those of Stowe and Schilke (27) and Grunwald *et al.* (15). Since paper- and thin-layer chromatograms of some *Nicotiana* extracts had suggested that other acidic growth hormones might be concealed at the location of IAA (3, 4) a gas chromatographic analysis was performed of those acidic indoles which are of physiological interest and which show the same or very similar R_F values on paper- and thin-layer chromatograms (table I). Gas chromatographic data obtained with 2 to 5 μg samples of methylated synthetic indole acids are summarized in table II and calculated according to the recommended practice for gas chromatography by Ettre (11). Relative retention values r are given with respect to ethylindole-3-acetate and the resolution is expressed as effective plate number N. All indoles gave responses with the SE-30 and QF-1 columns. Retention times varied between 5 min (IAA-ME) and 20 min (IAcA-ME). The crowding at the beginning of the chromatogram could not be eliminated by lowering flow rates or by lowering the temperature. In the latter situation the substances are insufficiently volatile (27).

Gas chromatography of naturally occurring indole esters in *Nicotiana* revealed several peaks which coincided in their location on chromatograms with those of the synthetic indole esters. Since these substances were not always found in the extracts of the investigated *Nicotiana* plants, each experiment was repeated at least 12 times and simultaneously

Table I. *R_F-Values of Indole Acids on Thin-Layer-Chromatograms, Run in n-butanol-H_2O-ammonia (10:10:1), Upper Phase*

Substance	R_F
Indole-3-acetic acid	0.35
Indole-3-carboxylic acid	0.22
Indole-3-propionic acid	0.44
Indole-3-butyric acid	0.55
Indole-3-lactic acid	0.48
Indole-3-acrylic acid	0.45

Table II. *Comparison of Retention Times (t'_R), Relative Retention Times (r), and Effective Plate Values (N) of 2 to 5 μg Samples of Various Indole Methyl-Esters on 3 % SE-30 and Silicone QF-1 Columns*

Column temperature: 190°; carrier gas flow rate: 30 ml/min.

Indole esters	3 % SE-30			3 % Silicone QF-1		
	t'_R	r	N	t'_R	r	N
Ethyl-indole-3-acetate	6.2	1.00	784	5.6	1.00	608
Methyl-indole-3-acetate	5.1	0.82	816	4.6	0.82	336
Methyl-indole-3-carboxylate	5.6	0.90	784	5.6	1.00	480
Methyl-indole-3-propionate	8.0	1.30	688	6.6	1.18	352
Methyl-indole-3-butyrate	9.7	1.58	994	8.3	1.48	576
Methyl-indole-3-lactate	10.0	1.64	970	9.6	1.72	848
Methyl-indole-5-hydroxyacetate	14.6	2.37	784	16.5	2.94	800
Methyl-indole-3-acrylate	17.1	2.82	702	20.8	3.71	752

compared with controls. In compensation experiments *Nicotiana* indole esters and the methylated synthetic controls were injected into the columns simultaneously. Overlapping of peak areas was indicative for the specificity of the extractable indole acids.

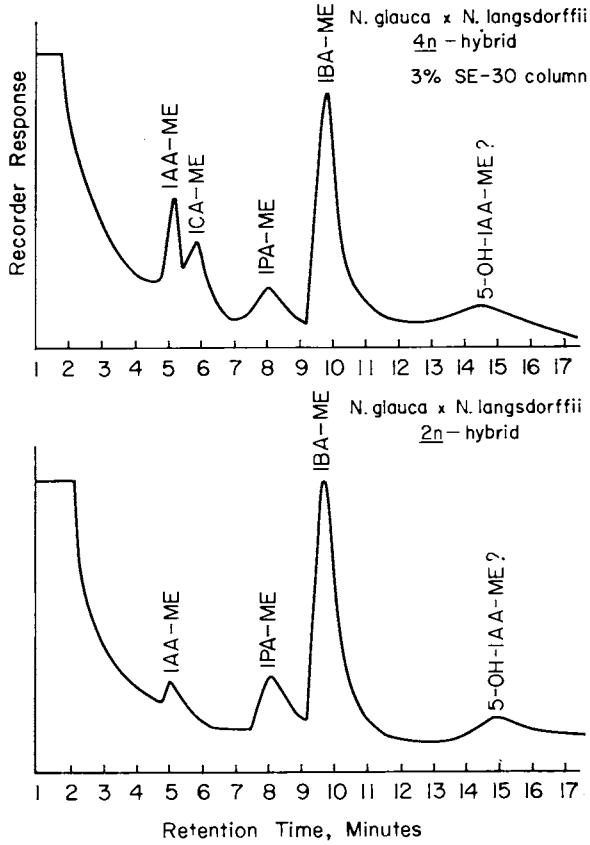

FIG. 1. Gas chromatogram of methylated indole acids derived from thin-layer chromatograms (run in *n*-butanol-ammonia-water) at R_F's 0.25 to 0.55 of *Nicotiana glauca* and *N. langsdorffii* extracts. Column: 3 % SE-30; temperature 190°. Argon flow rate: 30 ml/min. IAA-ME: indole-acetic acid methyl ester; ICA-ME: indole-carboxylic acid methyl ester; IPA-ME: indole-propionic acid methyl ester; IBA-ME: indole-butyric acid methyl ester; 5-OH-IAA-ME: 5-hydroxyindole-acetic acid methyl ester.

In *N. glauca* and *N. langsdorffii* 4 different methylated indole acids could be identified, *i.e.* ICA-ME, IAA-ME, IPA-ME, IBA-ME (fig 1). The corresponding acids are known to be of plant physiological significance since they act either as growth promoters in many plant tissues or are intermediates of IAA synthesis *in vivo* (5, 8, 10, 25,30).

The IAA content in *N. glauca* and *N. langsdorffii* is rather low (the recorder response indicates a concentration of about 0.7 μg IAA in 30 g fr tissue) but the indole-auxins ICA, IPA, and IBA are present at concentrations of about 1.0 μg each in 30 g tissue. Tissues of the tumor-prone 4n- and 2n-hybrids, however, show a considerable increase in the auxin IBA, the concentration of which lies around 1.8 to 2.2 μg per 30 g fresh tissue (fig 2).

On thin-layer chromatograms with *n*-butanol-H_2O-NH_3, the R_F of IBA lies considerably higher than that of the other investigated indole acids, *i.e.* at about 0.55 (table I). Therefore, purified fractions of 4n-hybrid extracts were divided into 2 groups, 1 containing the R_F sections 0.25 to 0.45 containing the indole acids ICA, IPA, and IAA (fraction I); the other R_F fraction 0.45 to 0.65, containing IBA and possibly other, unidentified indole compounds (fraction II). Figure 3 shows the gas chromatographic separation of the methyl esters of these indole acids. IBA-ME is present in the 0.45 to 0.65 thin-layer chromatogram fraction, whereas the other indole acids occur in the 0.25 to 0.45 fraction. Compounds with higher retention times (t'_R) than 9.7 (for IBA-ME) could be found in all investigated tissue extracts. Purification procedures of the plant extracts, thin-layer chromatography and esterification of the fractionated samples identifies them as indole esters. It may be suspected that broad peaks between retention times (t'_R) of 14 to 15 min (fig 2) represent the methyl ester of naturally occurring 5-OH-IAA. The low concentration of this substance in the plant material used, however, does not permit a clear identification of this auxin.

B) *Spectrophotofluorometry.* If a suspected acidic indole is separated and purified, spectrophotofluorometry can be employed to confirm the indolic nature of the compound (7, 16, 27). Therefore, col-

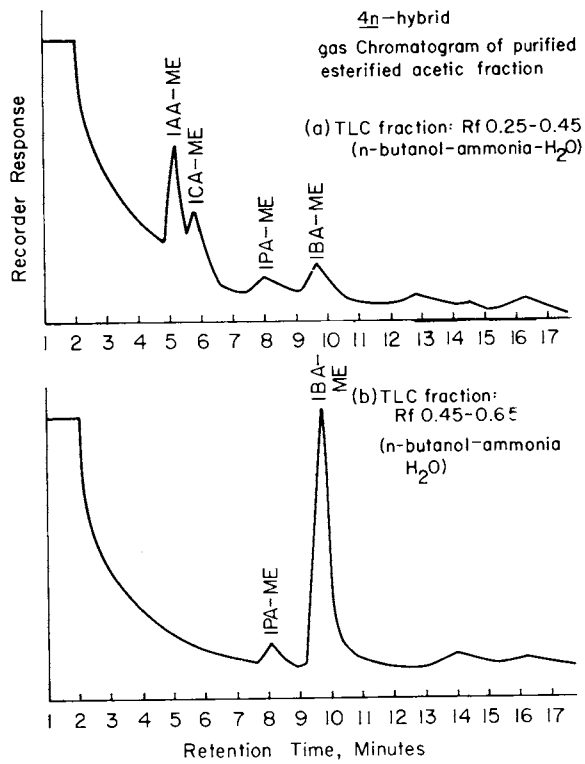

FIG. 2. Gas chromatograms of methylated indole acids, derived from thin-layer chromatograms at R_F's 0.25 to 0.55 of *N. glauca* × *N. langsdorffii* 4n and 2n hybrids. Specifications as in figure 1.

lected fractions from the gas chromatograms were transferred to quartz cuvettes and the measurements carried out on an Aminco-Bowman instrument. All investigated fractions had activation maxima in the 275 to 285 mμ range, with their major fluorescence peaks between 360 and 375 mμ. As anticipated, the spectrophotofluorometric spectra of the methyl esters

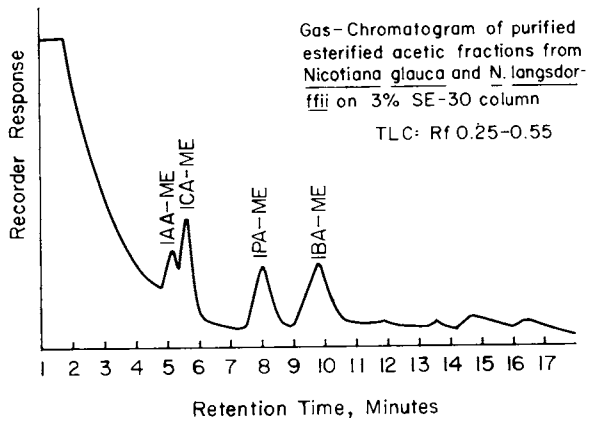

FIG. 3. Gas chromatogram of methylated indole acids, derived from thin-layer chromatograms of 4n hybrid extracts at a) R_F 0.25 to 0.45, and b) R_F 0.45 to 0.65. Specifications as in figure 1.

studied here were found to be almost identical to the indole acid spectra themselves. Spectrophotofluorometric measurements, therefore, confirmed the indolic nature of the isolated fractions. Actual identification of the compounds, however, rests most securely on the chromatographic properties cited above (15).

C) *Bioassay.* In several experiments, collected fractions of 4n hybrid extracts were directly transferred to agar blocks and measured by an *Avena* curvature biotest. Fraction I, containing the methyl esters of ICA, IAA, and IPA, gave an average coleoptile curvature of −8.5°, whereas the methyl esters of fraction II (containing IBA-ME) gave an average curvature of −5°. The relatively weak response of the *Avena* coleoptiles to these otherwise potent growth hormones is explained by the structural changes during methylation of these indole acids, since it has long been known that esterification of indole acids reduces their growth-promoting activities (29). Nevertheless, the growth response of *Avena* coleoptiles shows the auxinic nature of the investigated compounds.

Discussion

The gas chromatographic analysis of esterified indole acids in *Nicotiana* plants revealed several indole acids, IAA, ICA, IPA, and IBA. All 4 indoles could be isolated from *N. glauca* and *N. langsdorffii* extracts at low concentrations, whereas in the tumor-prone 4n- and 2n-hybrids the content of IBA was significantly increased. Although all identified indoles were present in the parental and the 4n hybrid tissues, 2n hybrids showed no detectable ICA. The significance of this possible deficiency remains to be studied in the future.

The synthesis of IAA from tryptophan is considered possibly to follow 2 different routes (10, 12) which include such intermediates as ICA and IPA together with several neutral indole compounds. The pathway of IAA synthesis in *Nicotiana* seems to follow the same proposed reaction chains. A separate pathway for the synthesis of ICA from tryptophan seems to be possible in some plant tissues, and ICA has been found by Klambt (17) in wheat coleoptiles and by Clarke *et al.* (8) in tomato crown gall tissue extracts. IPA with auxin activity in higher plants has been described by Fischer (13), Linser *et al.* (18) and Melchior (19).

Since the proposed pathway for IAA synthesis from tryptophan does not include IBA as an intermediate it may be that this auxin is the product of a separate auxin-producing pathway in *Nicotiana*, in which case it may be a storage form of IAA. IBA has been found to be a naturally occurring auxin in another one of the *Solanaceae*, that is, in *Solanum tuberosum* (5). The evidence for the occurrence of IBA in potato tissues was based on paper-chromatographic separations. The present identification of IBA in *Nicotiana* suggests a wider distribution of this auxin in higher plants.

Acknowledgments

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Conversion of Indole-3-butyric Acid to Indole-3-acetic Acid by Cuttings of Grapevine (*Vitis vinifera*) and Olive (*Olea europea*)[1]

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The metabolism of indolebutyric acid (IBA) in hardwood cuttings of grapevine (*Vitis vinifera* cv. Perlette) and green cuttings of olive (*Olea europea* cvs. Manzanillo, Kalamata and Koroneiki) was investigated. Radioactive IBA which was synthesized in our laboratory was used in these studies. Cuttings of both olive and grapevine converted IBA to IAA. The identity of IAA was confirmed by high performance liquid chromatography and gas-liquid chromatography.

The stability of IBA, its slow transport from the site of application at the base of the cutting and its conversion to IAA in the cutting are probably the factors which make this compound a good root promoter.

Key words: GLC — Grapevine — HPLC — IAA — IBA — Olive.

Natural and synthetic auxins are known to induce root initiation in stem cuttings (Cooper 935, Gautheret 1969, Thimann and Koepfle 1935). The use of auxins to stimulate rooting in uttings was the first practical application of plant hormones in agriculture. The value of oot-promoting substances during vegetative plant propagation has been extensively studied, ut only a few reports are available concerning the mode of action of these substances. Fawcett t al. (1960) demonstrated the conversion of the synthetic auxin IBA to the naturally-occurring ne, IAA. They exposed wheat coleoptile and pea stem tissue to IBA solutions and subsequently lentified IAA in the tissues and the residual solution. With the availability of radioactive IBA Cohen and Schulze 1981) and methods of IAA extraction and identification (Epstein and Cohen 981), it became possible to determine this conversion in hardwood and green cuttings by llowing the conversion of IBA-^{14}C to IAA-^{14}C.

Materials and Methods

Single-bud cuttings (one internode) of grapevine (*Vitis vinifera* cv. Perlette) cuttings were ken from a 3 year-old vineyard. For experiments with olive (*Olea europea*), green cuttings with aves of cv. Manzanillo (easy-to-root) and cv. Kalamata (hard-to-root), were used. The rooting edium consisted of a 10-cm-deep Dutch peat and shredded polysterene foam mixture, 2 : 1.

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Abbreviations: GLC, gas-liquid chromatography; HPLC, high performance liquid chromatography; IBA, indolebutyric acid; TLC, thin layer chromatography.

Rooting was done under constant fluorescent light at 25°C, under mist irrigation. Before plant ing the cuttings their lower surface was cut again and 10 μl of [2-^{14}C]indole-3-butyric aci (12,000 cpm) was applied to the fresh cut. The cuttings were then dipped in a commercia rooting powder containing 0.5% IBA. [2-^{14}C]indole-3-butyric acid was synthesized fron indole-2-^{14}C (48 mCi/mmole; CEA, Gif Sur Yvette, France) and butyrolactone, as describe by Cohen and Schulze (1981).

Extraction of IAA and IBA—For identification of IAA and IBA, 20 cuttings of grapevine o olive were removed from the medium 26 days after planting and washed with water. Th bases (2 cm section) were extracted with an Ultra Turrax in 70% acetone. The radioactiv extracts were filtered, evaporated to the aqueous phase, brought to pH 2.5 with 5 M sulfuric acid and extracted three times with ethyl acetate. The extracts were pooled, dried, evaporated t dryness, and the residue was taken with 1 ml methanol for sephadex LH-20 column chromato graphy and TLC.

IAA transport studies—Six olive cuttings were divided into base (site of application), node (I, II and III) and leaves, and thereafter extracted separately as above. The extracts wer filtered and evaporated to the aqueous phase and samples were taken for radioactivity countin and TLC. Sephadex LH-20 column chromatography was done as described previously (Bandurski and Schulze 1974). The column (0.6 × 22 cm) was prepared with 50% 2 propanol/water (v/v). One half ml of the cuttings' extracts was applied to the column. Elutio was done with 50% 2-propanol/water (v/v) at a rate of 0.1 ml·min^{-1}. Two ml fractions wer collected. Tubes containing radioactivity were pooled, evaporated to dryness, and taken u with 0.5 ml of methanol for TLC and gas chromatography. Thin-layer chromatography wa done on Kieselgel 60 F254 plates with chloroform : acetic acid, (95 : 5, v/v) as solvent. Th sample was applied to the plate as an 8-cm long band with IAA and IBA co-spotted as standards The location of IAA and IBA were marked under UV-light. The bands corresponding to thei location (Rf 0.32 and 0.45 for IAA and IBA, respectively), as well as bands under and above were scraped off the plates and counted in a scintillation counter.

Detection by HPLC—IAA and IBA from the column chromatography were further separated and identified by HPLC. A micro-processor-controlled Varian model 5000 was used. Chro matography was carried out on a Micropac C-18 reverse phase column using 35% methanol and 65% of 5% aqueous tetrahydrofuran at a flow rate of 2 ml·min^{-1}.

Detection by GLC—Further information on the identity of IAA was obtained by gas-liquid chromatography. The IAA fraction eluted by HPLC was further purified by TLC and deriv atized by α-bromo-pentafluorotoluene (Epstein and Cohen 1981). For electron-capture detection, a Packard model 419 electron capture detector was used. The injector port and detector were kept at 250°C, and the column oven at 230°C. Nitrogen was used as a carrier gas, at 30 ml·min^{-1}. The column was 1.3 m × 4 mm glass packed with 1% OV-17 on Gas-Chrom Q. The detector was operated in pulse mode with 10 μsec pulse period.

Results

Cuttings of olive developed large calluses and roots two weeks after treatment. Easy to root cultivars rooted after two weeks, while hard to root cuttings developed only calluses and eventually dried out. The transport studies showed that twenty-six days after planting about half of the recovered radioactivity remained at the base of the cuttings (site of application), and about a third was transported to the first node (Table 1). There was some indication that more of the label moved from the base to nodes II and III in cv. Kalamata. Only a little radio activity was found in the leaves of both cultivars. In another experiment 10 cm-long green cuttings of cv. Koroneiki (easy to root) and cv. Kalamata (hard to root) were treated with

Table 1 Distribution of radioactivity (% of total radioactivity recovered and applied) in olive cuttings of cv. Manzanillo (easy-to-root) and cv. Kalamata (hard-to-root)

Fraction	Manzanillo		Kalamata	
	% of total radioactivity recovered	% of total radioactivity applied	% of total radioactivity recovered	% of total radioactivity applied
Base	50.6±4.4	19.0±1.7	42.1±10.1	21.9±2.5
I	35.5±6.3	13.8±2.1	36.7± 8.3	21.3±2.4
II	7.3±3.2	4.5±1.3	13.4± 7.1	7.3±1.6
III	3.3±1.9	1.1±0.3	5.2± 5.5	5.0±1.8
Leaves	3.3±1.1	1.5±0.4	2.6± 1.3	1.9±0.9

Each figure is the mean of five replicates±SD. Extraction and counting were done 26 days after application of IBA-^{14}C to the base of the cuttings.

IBA-^{14}C and rooted as described above. Six cuttings of each cultivar were removed 8, 18, 27, and 78 days after planting. The lower half, upper half and leaves were extracted separately, as in the previous experiment and the total radioactivity in each plant part was determined. Most of the recovered radioactivity (about 90%) was found in the lower part of the cuttings (Table 2). However, after 78 days, about 20% of the label was detected in the upper part of Koroneiki cuttings, but there was very little transport to leaves or roots.

Thin layer chromatography of the radioactive extracts from both olive cultivars taken at various times after planting showed that with time most of the recovered radioactivity was found in the form of IAA-^{14}C (Fig. 1). In both cultivars 20–30% of the recovered label was found in IAA after 3 days and after a month about 70% of the radioactivity was in IAA. In Koroneiki 2 months after IBA-^{14}C application only 10% of the recovered radioactivity was still in IBA-^{14}C while 90% was converted to IAA-^{14}C. The conversion of IBA to IAA was somewhat faster in the hard to root cv. Kalamata than in Koroneiki. Since cv. Kalamata did not root, the cuttings dried and we could not follow the IBA metabolism in these cuttings for more than one month.

Similar results were obtained with the hardwood grapevine cuttings cv. Perlette (Table 3).

Table 2 Distribution of radioactivity (% of total radioactivity recovered and applied) in olive cuttings of cv. Koroneiki (easy-to-root) and cv. Kalamata (hard-to-root)

Source	% of total radioactivity recovered				% of total radioactivity applied			
	Day after treatment				Day after treatment			
	8	18	27	78	8	18	27	78
Koroneiki								
Lower half	93.0	92.0	92.0	70.2	10.3	7.8	14.5	15.8
Upper half	3.0	5.7	5.0	22.6	0.3	0.5	0.8	5.0
Leaves	4.0	2.3	3.0	2.4	0.5	0.2	0.5	0.6
Roots	–	–	–	4.8	–	–	–	1.1
Kalamata								
Lower half	87.8	95.0	93.0	—	8.5	22.2	15.6	—
Upper half	3.5	3.0	3.0	—	0.4	0.8	0.4	—
Leaves	8.7	2.0	5.0	—	1.0	0.4	0.8	—
Roots	–	–	–	—	–	–	–	—

Six cuttings were used. Total radioactivity was determined at various periods following the application of IBA-^{14}C to the base of the cuttings.

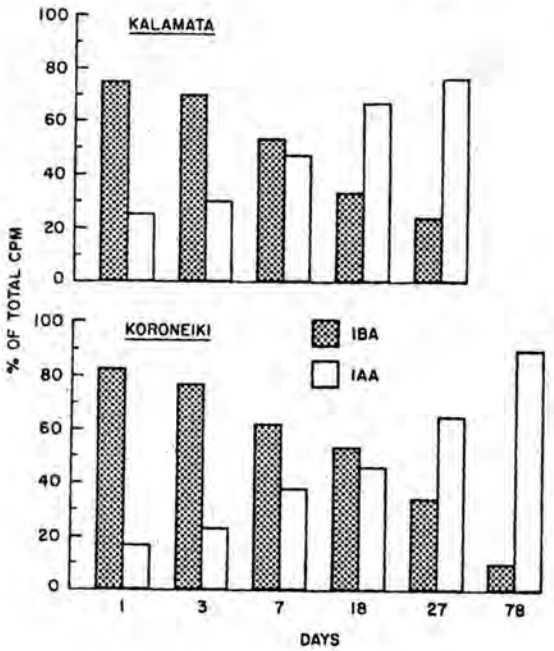

Fig. 1 Distribution of radioactive IBA and IAA in extracts of olive cuttings various times after application of IBA-^{14}C. Radioactivity was determined after TLC in chloroform : acetic acid (95 : 5, v/v) by scraping off the silica gel from the plate at the appropriate Rf zone and counting it in a scintillation counter. Results are expressed as % of total activity of IAA and IBA recovered from the TLC.

In four seperate experiments 50–80% of the recovered label was found in IAA and the variability was probably due to differences in cuttings and growth conditions.

Chromatography of the grape vine extract on LH-20 Sephadex column with 50% 2-isopropanol water (v/v) resulted in two distinct peaks, one between 140 and 170 ml and the other between 180 and 250 ml. These peaks corresponded to elution times of the standard IBA and IAA, respectively.

IAA and IBA were further purified by HPLC. After chromatography tubes containing radioactive IAA and IBA were pooled. Under the conditions described above, IAA was eluted between 6 and 13 ml and IBA between 18 and 26 ml, the same as authentic samples. The IAA and IBA fractions were concentrated in vacuum and used for the GLC studies.

Final identification of IAA by GLC is shown in Fig. 2 and 3. Figure 2 shows a chromatogram obtained by injection of the sample extracted from the cuttings after application of IBA. The pentafluortoluene deriative of the putative IAA is represented on the graph by a peak at

Table 3 Radioactivity (cpm) of grapevine-cutting extracts after TLC in chloroform : acetic acid (95 : 5, v/v)

Rf	Radioactivity (cpm)			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
0.26	167	102	36	37
0.32 (IAA)	370	390	113	535
0.39	24	37	9	14
0.45 (IBA)	233	221	25	14
0.55	15	24	—	—

The radioactivity of the appropriate zones was determined by scraping off the silica gel from the plate at the appropriate Rf zone and counting it in a scintillation counter. Extraction was done 27 days after application of radioactive IBA.

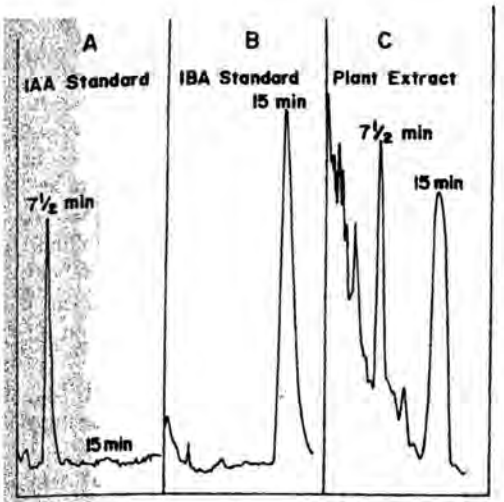

Fig. 2

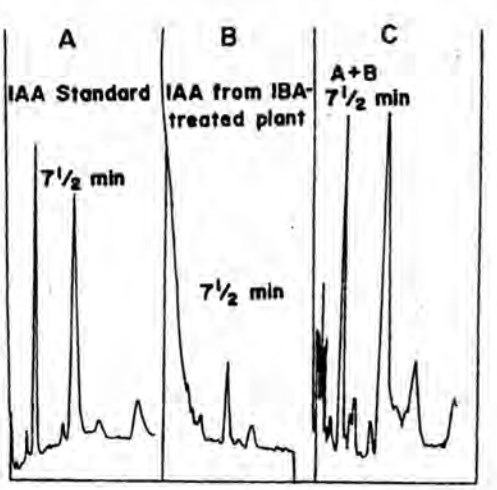

Fig. 3

Fig. 2. GLC profile obtained with pentafluorbenzene derivatives of standards of IAA and IBA and the extract of IBA-treated grapevine cuttings.

Fig. 3. GLC chromatograms of pentafluorobenzene derivatives of standard IAA, IAA from plant extract, and a mixture of the two.

Rt=7.5. This peak is identical to the Rt value of authentic IAA, and was enhanced by the addition of known amounts of synthetic IAA which was derivatized the same way as the sample extracts (Fig. 3).

Alternatively, the conversion of IBA to IAA was demonstrated by another approach. Radioactive IBA (636,800 dpm) was applied to 20 cuttings of grapevine and the cuttings were kept in rooting medium as described above. After two weeks, 2 cm of the cuttings' bases were homogenized in a Waring Blender in 500 ml 70% acetone containing 3 mg IAA. The homogenate was left overnight and then filtered through Eaton-Dikeman 515 paper to remove insoluble residues. The filtrate was then reduced to an aqueous phase of 80 ml in vacuum. This was acidified with 3 M sulfuric acid to pH 2.5 and partitioned 3 times against an equal volume of chloroform. Pooled chloroform phases were dried over anhydrous sodium sulfate, reduced in vacuum, then dissolved in 1 ml of 50% 2-propanol/water (v/v) and placed on a 1.5×12-cm DEAE-Sephadex-acetate A-25 (Sigma) column, equilibrated with 50% 2-propanol/water (v/v). The column was washed with 50% 2-propanol/water (v/v) until the eluent was color-free, then the sample was eluted with a linear gradient of 0–5% acetic acid in 50% 2-propanol/water (v/v). IAA-containing tubes were located by spotting a 5 μl aliquote from each tube on silica gel 60 TLC plate, developing the plates in chloroform : methanol : water (85 : 14 : 1, v/v/v) and then using Ehmann's reagent (Ehmann 1977) for IAA detection. IAA was eluted from the column between 88–114 ml. Fractions containing IAA were pooled and evaporated to dryness. The residue was dissolved in 100 μl of 50% methanol/water (v/v) and a sample was taken for radioactive counting. Total radioactivity of the IAA fraction was 18,000 dpm. The rest of the fraction containing the IAA was injected onto HPLC column of 4.6 mm×25 cm Partisil 10 ODS-3 (Whatman). The mobile phase was 30% methanol/water (v/v) containing 1% acetic

acid, with a flow rate of 1 ml·min^{-1}. Collected fractions were tested for IAA on TLC as described above. IAA was eluted between 19–21 ml. Total radioactivity was found to be 2,800 dpm.

Discussion

Using radioactive IBA, we were able to show that both hardwood cuttings of grapevine and green cuttings of olive metabolized the synthetic auxin, IBA, to the natural one, IAA. The formation of IAA was confirmed by comparison with authentic IAA on TLC column of LH-20 HPLC and GLC. In all these systems a radioactive compound (which was metabolised from the applied IBA) was found at the same Rf or Rt as the IAA. In one experiment, the addition of a large amount of cold IAA to the extract allowed us to locate the IAA on TLC plates by the use of an appropriate reagent and then to determine its radioactivity. We used 3 methods of purification in this experiment: TLC, DEAE-Sephadex column and HPLC on reverse phase column. After each step we found that IAA was radioactive thus reducing the possibility that the radioactive compound was other than IAA.

The conversion of IBA to IAA was somewhat faster in the hard-to-root (Kalamata) than in the easy-to-root (Koroneiki) cuttings of olive. This difference was noted during a period of 27 days after the labeled IBA application. After 78 days most of the recovered label (90%) in the extract from cv. Koroneiki was in IAA-^{14}C. The Kalamata which did not root dried out at this stage.

Some workers have shown the importance of endogenous IAA level on the rooting process. Dunberg et al. (1981) found that IAA content at the bases of IBA-treated cuttings of *Pinus sylvestris* was three times higher than in untreated cuttings. They did not find differences in IAA metabolism or transport between the treated and untreated cuttings and concluded that the applied IBA was converted to IAA by the cuttings. Brunner and Mezel (1976), Hemberg (1954), Hess (1959), Odom and Carpenter (1965) and Tyce (1957) have shown the importance of IAA level in the cuttings for rooting. Nakano et al. (1980) showed that auxin activity at the base of the cuttings diminished during propagation, and it was lower in the well-rooted than in the unrooted cuttings. They assumed that the auxin might have been used for root differentiation. Thus, it is suggested that the promoting effect of IBA on rooting is mainly the result of its conversion to IAA by the cutting.

Most of the IBA, as well as the IAA which was synthesized by the cuttings from IBA, remained at the base of the cutting and did not move upward. Similar results were obtained by Strydon and Hartmann (1960) using radioactive IAA. Very little radioactivity was found in the leaves of the plants used in the present study and appreciable differences were noted between the easy- and hard-to-root varieties.

IAA is oxidised readily in the plant by peroxidases (Galston and Hillman 1961). IBA is probably a good rooting promoter because it is not oxidised by peroxidases (Gorter 1961). It remains at the base of the cutting and releases free IAA which is needed for the rooting process.

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The Five "Classical" Plant Hormones

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INTRODUCTION

It is 60 years since Went and Thimann (1937) published their classic book *Phytohormones*. At that time, the term phytohormone was synonymous with auxin, although the existence of other phytohormones, such as cell division factors, was anticipated on the basis of physiological experiments. It is impressive that aside from some confusion about the structure of auxin, many of the basic phenomena of auxin physiology were already known at that time. It is equally impressive that much auxin biology, including the Cholodny-Went hypothesis (Went and Thimann, 1937) regarding the role of auxin in mediating gravi- and phototropism, the pathway of auxin biosynthesis, and the mechanism by which auxin causes cell wall loosening, remains controversial.

Since 1937, gibberellin (GA), ethylene, cytokinin, and abscisic acid (ABA) have joined auxin as phytohormones, and together, they are regarded as the "classical five" (Figure 1). This group is expected to grow as the hormonal functions of other compounds are recognized and as new hormones are discovered (see Creelman and Mullet, 1997, in this issue). As is evident from this short review, recent progress on hormone biosynthesis and on hormonal transduction pathways has been impressive. Also evident is that there are many blanks still to be filled in. With the application of the powerful new techniques of chemical analysis and molecular genetics, the rate at which new discoveries are made will continue to accelerate. It's a great time to be a plant hormonologist!

CHEMISTRY AND BIOSYNTHESIS OF HORMONES

Clearly, the amount of any compound, including hormones, in an organ of a plant is determined by the combined rates of its biosynthesis, breakdown, import, and export. The last two aspects are not considered in the following discussion of how the endogenous pool sizes of the five classical hormones are regulated. Rather, we focus on the biosynthetic pathways, the deactivation reactions, and the regulatory mechanisms involved in these processes.

During the past 25 years, the standards set for natural-product chemistry have also been applied to plant hormone research. Instead of "measuring" hormones by bioassays, unambiguous physical–chemical methods for the identification and measurement of hormones have been developed (reviewed in Hedden, 1993). The accuracy and facility of quantitative measurements have been improved by the availability of isotopically labeled versions (with ^{2}H, ^{13}C, or ^{15}N) of the hormones for use as internal standards.

Molecular genetics is another discipline that has made it possible to solve problems in hormone physiology that were hitherto intractable. Hormones are present in plants in very small amounts. Moreover, their biosynthetic and catabolic enzymes are low-abundance proteins, which, in most cases, cannot be isolated and purified by classical biochemical methods. However, when the identification of a mutant leads to the cloning of a gene, that gene can be expressed as a fusion protein with which the catalytic function can be determined (e.g., Sun and Kamiya, 1994; Xu et al., 1995; Schwartz et al., 1997b).

Auxin

The primary auxin in plants is indole-3-acetic acid (IAA; Figure 1). Although other compounds with auxin activity, such as indole-3-butyric acid, phenyl acetic acid, and 4-chloro-IAA, are also present in plants (reviewed in Normanly et al., 1995), little is known about their physiological role. For many years, it has been assumed that tryptophan is the precursor of IAA. This has recently been confirmed in seedlings of *Phaseolus vulgaris* with stable isotope labeling studies (Bialek et al., 1992). Three routes for IAA biosynthesis from tryptophan via indole-3-pyruvic acid, tryptamine, or indole-3-acetonitrile have been proposed. The latter precursor is found primarily in the Cruciferae and may be derived from

This review is dedicated to the memories of James Bonner, Anton Lang, Kenneth Thimann, and Philip Wareing, pioneers in plant hormone research, who died during the past year.

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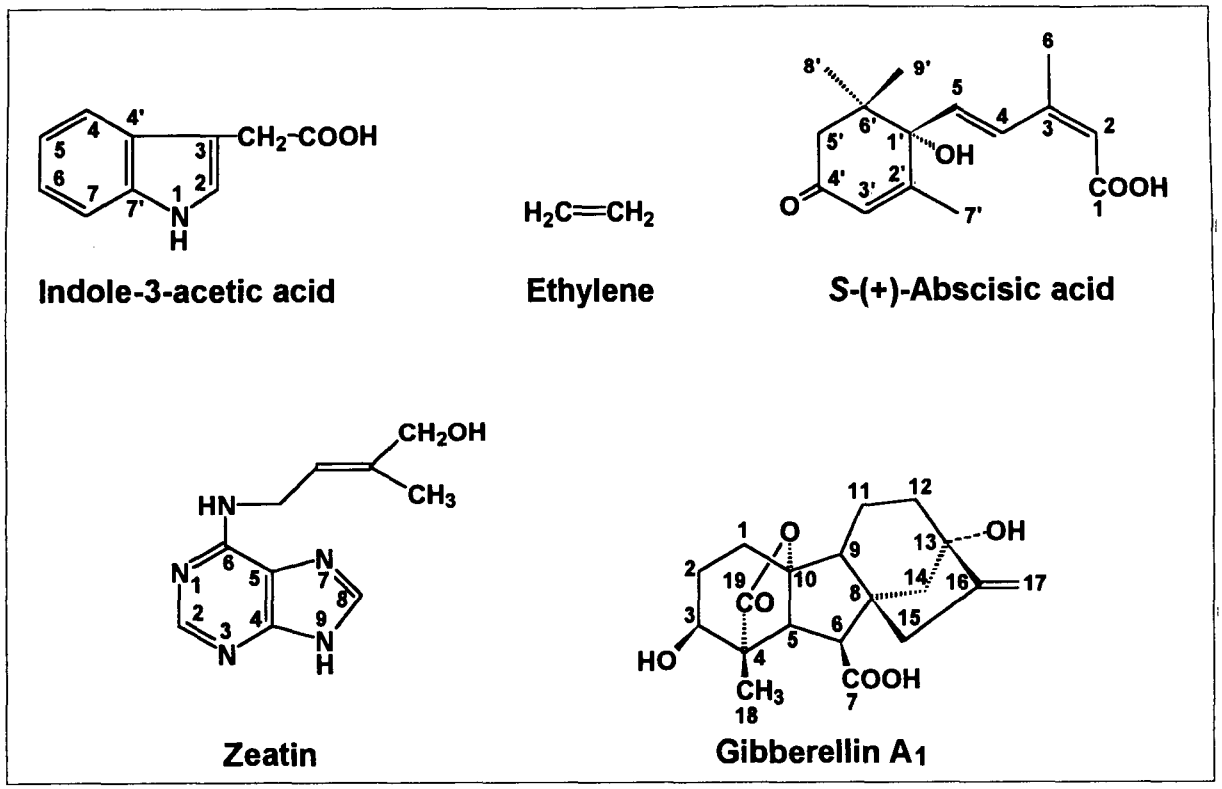

Figure 1. Structures of Representatives of the Five Classical Plant Hormones.

Shown are indole-3-acetic acid, ethylene, abscisic acid, zeatin, and gibberellin A_1.

indoleglucosinolates (Normanly et al., 1995). Four genes encoding nitrilase, which converts indole-3-acetonitrile to IAA, have been cloned in Arabidopsis. These four genes are differentially regulated (Bartel and Fink, 1994).

Work with tryptophan auxotrophic mutants has established that IAA biosynthesis can also take place via a tryptophan-independent route. For example, the *orange pericarp* mutant in maize does not produce tryptophan but accumulates IAA to levels 50-fold higher than in the wild type (Wright et al., 1991). Tryptophan auxotrophs in Arabidopsis also accumulate more IAA than do wild-type plants. On the basis of these data, it was proposed that IAA can be synthesized through a branch point of the tryptophan biosynthetic pathway at indole or indole-glycerol phosphate (Normanly et al., 1995). Supporting this idea is the finding that in a cell-free system from immature maize endosperm, radioactive indole is converted to IAA (Rekoslavskaya and Bandurski, 1994).

Certain bacteria and plant cells transformed with *Agrobacterium tumefaciens* synthesize IAA via a unique pathway in which tryptophan is converted to IAA in two steps. The first enzyme, tryptophan monooxygenase, converts tryptophan to indole-3-acetamide, which in turn is converted to IAA by indole-3-acetamide hydrolase. The genes encoding these enzymes have been used to alter IAA levels in transgenic plants (Klee and Romano, 1994).

IAA occurs not only in the free form but also conjugated to amino acids, peptides, or carbohydrates. These IAA conjugates are biologically inactive and appear to serve functions as IAA storage forms in seeds and hormonal homeostasis. The *iaglu* gene in maize, which encodes an enzyme that esterifies IAA to glucose, has been cloned (Szerszen et al., 1994). In Arabidopsis, a gene family that encodes IAA conjugate hydrolases has been identified (Bartel, 1997).

Until recently, IAA catabolism was thought to occur via oxidative decarboxylation (i.e., through the action of an IAA oxidase). However, the major catabolic route of IAA in vivo now appears to be oxidation to oxindole-3-acetic acid and subsequent glycosylation through an added 7-hydroxyl (reviewed in Normanly et al., 1995). Another catabolic pathway is via IAA-acetylaspartate to dioxindole-3-acetylaspartate-3-*O*-glucoside.

GAs

Since the first GA from a higher plant, GA_1 (Figure 1), was identified 40 years ago, 112 GAs have been identified to date (Hisamatsu et al., 1997). Efforts to determine the physiological roles of GA and to elucidate the biosynthetic pathway have been greatly facilitated by the availability of GA-deficient (i.e., dwarf) mutants. Metabolic studies have been conducted with systems that are rich sources of GAs, such as the fungus *Gibberella fujikuroi* and immature seeds of pumpkin, pea, and bean. However, maize is the only higher plant in which the entire biosynthetic pathway has been demonstrated in vegetative tissues by feeding various intermediates (Suzuki et al., 1992; Kobayashi et al., 1996). These and other studies have shown that the GA biosynthetic pathway can be divided into three stages (Figure 2; reviewed in Graebe, 1988; Hedden and Kamiya, 1997; MacMillan, 1997). These stages are considered below.

Stage 1: From Geranylgeranyl Diphosphate to ent-*Kaurene*

The first committed step in GA biosynthesis is the cyclization of geranylgeranyl diphosphate to *ent*-copalyl diphosphate, which in turn is converted to *ent*-kaurene (Figure 2A). The enzymes that catalyze these reactions have been called *ent*-kaurene synthase A and B, respectively, but MacMillan (1997) has proposed the more appropriate terms *ent*-copalyl diphosphate synthase and *ent*-kaurene synthase. The genes encoding these enzymes have been cloned from Arabidopsis (*GA1*; Sun and Kamiya, 1994) and pumpkin endosperm (Yamaguchi et al., 1996), respectively. Biochemical evidence indicates that both enzymes are located in proplastids of meristematic shoot tissues but not in mature chloroplasts (Aach et al., 1997). Because conversions in stage 3 of the pathway can take place in mature leaves, it is likely that pathway intermediates move between different tissues and organs. In Arabidopsis, the expression of *GA1* is highly regulated during growth and development. Promoter studies with β-glucuronidase as reporter gene indicate that *GA1* expression is highest in shoot apices, root tips, and the vascular tissue of leaves (Silverstone et al., 1997).

Stage 2: From ent-*Kaurene to GA_{12}-Aldehyde*

The enzymes in the second stage of the pathway are membrane-bound P450 monooxygenases, which are thought to be located in the endoplasmic reticulum. The sequential oxidation of C-19 of *ent*-kaurene via *ent*-kaurenol and *ent*-kaurenal to *ent*-kaurenoic acid is probably catalyzed by a single enzyme, the activity of which is impaired in the *ga3* mutant of Arabidopsis (J.A.D. Zeevaart, unpublished results). *ent*-Kaurenoic acid is further oxidized to *ent*-7α-kaurenoic acid (Figure 2B). Contraction of the B-ring with extrusion of C-7 gives GA_{12}-aldehyde. The *Dwarf3* (*D3*) gene of maize encodes a P450 monooxygenase, but it is not known which step in stage 2 of the pathway is catalyzed by the D3 protein (Winkler and Helentjaris, 1995).

Stage 3: From GA_{12}-Aldehyde to Various GAs

The first step in stage 3 of the pathway involves oxidation of GA_{12}-aldehyde to GA_{12}. Further metabolism of GA_{12} varies among species or organs of the same species with respect to the position and sequence of oxidative steps. The early-13 hydroxylation pathway, which involves hydroxylation at C-13 to give GA_{53}, is common in higher plants. After C-13 hydroxylation, C-20 is successively oxidized and eliminated by the multifunctional enzyme GA 20-oxidase via GA_{44} and GA_{19} to the C_{19}-GA, GA_{20} (Figure 2C). Finally, 3β-hydroxylase converts GA_{20} to the bioactive GA_1. All of these oxidative steps are catalyzed by dioxygenases that require 2-oxoglutarate and molecular oxygen as cosubstrates and Fe^{2+} and ascorbate as cofactors.

GA 20-oxidases have been cloned and expressed from a number of species (e.g., Lange et al., 1994; Phillips et al., 1995; Xu et al., 1995; Martin et al., 1996; Wu et al., 1996), although to date the gene encoding 3β-hydroxylase has been cloned only from Arabidopsis (*GA4*; Chiang et al., 1995). Expression of both the *GA4* and *GA5* (which encodes GA 20-oxidase) genes in Arabidopsis is subject to negative feedback regulation (Chiang et al., 1995; Phillips et al., 1995). In spinach, GA 20-oxidase activity is under photoperiodic control (Wu et al., 1996; see also Kreps and Kay, 1997, in this issue).

Of all the known GAs, only a few are bioactive per se, whereas the others are precursors or deactivated GAs. A 3β-hydroxyl group (as in GA_1 and GA_4) is required for activity, as was originally demonstrated with the dwarf *le* mutant of pea (Ingram et al., 1984) and *dwarf1* (*d1*) of maize (Spray et al., 1984). These mutants are impaired in 3β-hydroxylase activity, and normal growth can be restored by GA_1 but not by GA_{20}.

At the end of the pathway, bioactive GAs are generally deactivated by 2β-hydroxylation. For example, GA_1 is converted to GA_8 and GA_4 is converted to GA_{34}. Conjugation to glucosyl esters or glucosides takes place predominantly in maturing seeds. There is only one example of a deactivation mutant, the *slender* (*sln*) mutant in pea, in which 2β-hydroxylation is blocked and GA_{20} accumulates in maturing seeds (Ross et al., 1995).

Cytokinins

Naturally occurring cytokinins are N^6-substituted adenine derivatives (Figure 1). In addition to higher plants, several bacteria, including *Agrobacterium*, produce cytokinins (reviewed in Morris, 1986; Gaudin et al., 1994). The key biosynthetic

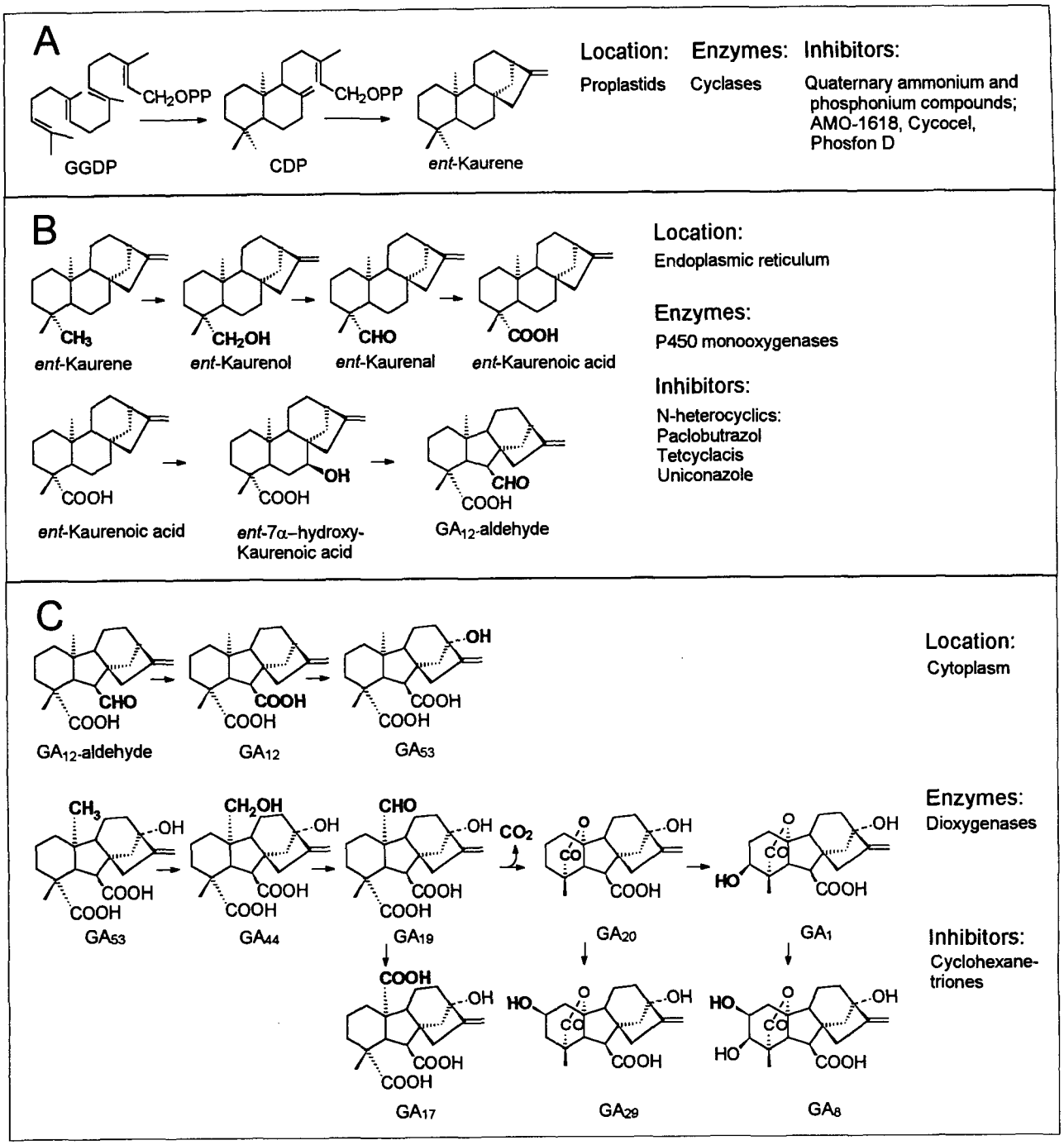

Figure 2. GA Biosynthetic Pathway.

(A) Stage 1: conversion of geranylgeranyl diphosphate (GGDP) to *ent*-kaurene. CDP, *ent*-copalyl diphosphate.
(B) Stage 2: from *ent*-kaurene to GA_{12}-aldehyde.
(C) Stage 3: represented here by the early-13 hydroxylation pathway from GA_{12}-aldehyde to GA_1 and GA_8.
For each stage, the nature of the enzymes, their location, and specific inhibitors are indicated. In **(B)** and **(C)**, the modifications taking place at each step are highlighted in boldface.

step in *Agrobacterium* is the addition of an isopentenyl group from isopentenyl diphosphate to N^6 of AMP, which is catalyzed by AMP–isopentenyl transferase (IPT). A similar enzyme activity has also been observed in extracts from plant sources (Blackwell and Horgan, 1994; Chen and Ertl, 1994), but because of its instability, the enzyme has only been partially purified (Chen and Ertl, 1994). The *IPT* gene from *Agrobacterium* has been cloned and expressed in transgenic plants (Klee and Romano, 1994). However, there are no reports of plant DNA sequences with similarity to the bacterial *IPT* genes (Binns, 1994).

Isopentenyladenosine 5′-monophosphate is the precursor of all other forms of cytokinins. Through hydroxylation of the isopentenyl side chain and reduction of the double bond, the ribotides of zeatin and dihydrozeatin are formed. It is generally thought that the free bases, such as isopentenyl adenine, zeatin, and dihydrozeatin, are the active forms of cytokinins. Cytokinins with a hydroxylated side chain can be glycosylated to form the *O*-glucoside or *O*-xyloside. These reactions are reversible, because *O*-glycosylated cytokinins have biological activity. Zeatin *O*-xylosyl transferase has been isolated from bean embryos, and antibodies have been prepared. The enzyme is predominantly localized in the endosperm (Martin et al., 1993).

Cytokinins are inactivated irreversibly by two different reactions: formation of N-conjugates with glucose at the 7- or 9-positions or with alanine at the 9-position and the oxidative cleavage of the N^6 side chain of the cytokinin substrate by cytokinin oxidase. The substrates for cytokinin oxidase are isopentenyladenine, zeatin, and their ribosides. By contrast, dihydrozeatin is resistant to cytokinin oxidase. Tobacco plants transformed with the *IPT* gene, which have an elevated cytokinin level, exhibit an increase in cytokinin oxidase activity in both leaves and roots (Motyka et al., 1996). These results indicate that cytokinin oxidase is induced by its own substrate and thus plays a role in regulating cytokinin levels in plants. Genetic manipulation of cytokinin oxidase may provide a strategy through which cytokinin levels can be modified.

ABA

Work on ABA (Figure 1) is a good example of how a combination of genetic, molecular, and biochemical approaches can lead to the elucidation of a complex biosynthetic pathway. Early on, the similarity in structure between ABA and the end groups of certain carotenoids led to the proposal that ABA may be a breakdown product of carotenoids, with xanthoxin as an intermediate. This idea was supported by the finding that plants that do not accumulate carotenoids (either because of mutation or treatment with inhibitors) also lack ABA. Furthermore, labeling studies with $^{18}O_2$ established that one ^{18}O atom is rapidly incorporated into the carboxyl group of ABA, indicating that there is a large precursor pool (i.e., carotenoids) that already contains the oxygens on the ring of the ABA molecule (Zeevaart et al., 1991). Finally, in etiolated leaves and roots, which have low levels of carotenoids, a 1:1 stoichiometry was found between the disappearance of violaxanthin and neoxanthin and the appearance of ABA and its catabolites (Li and Walton, 1990; Parry et al., 1992).

The ABA-deficient *aba1* mutant of Arabidopsis is blocked in the epoxidation of zeaxanthin to antheraxanthin and violaxanthin, indicating that the epoxycarotenoids violaxanthin and neoxanthin are essential for ABA production (Rock and Zeevaart, 1991). The *aba2* mutant of *Nicotiana plumbaginifolia* is orthologous with *aba1* of Arabidopsis and has been cloned. The corresponding fusion protein has zeaxanthin epoxidase activity (Marin et al., 1996).

The last two steps in the pathway, from xanthoxin to ABA-aldehyde to ABA, are catalyzed by constitutively expressed enzymes (Sindhu and Walton, 1988; Schwartz et al., 1997a), with the result that the level of xanthoxin in leaves is always very low relative to ABA (Parry et al., 1990). The *aba2* mutant in Arabidopsis is the only known mutant for the conversion of xanthoxin to ABA-aldehyde. By contrast, mutants for the final step, ABA-aldehyde to ABA, have been found in a number of species (reviewed in Taylor, 1991). In some cases (e.g., *nar2a* in barley, *flacca* in tomato, and *aba3* in Arabidopsis), the lesion is not in the aldehyde oxidase apoprotein but in the molybdenum cofactor that is required by the enzyme.

ABA biosynthesis increases when plant cells lose turgor (reviewed in Zeevaart and Creelman, 1988), raising the question of which step in the pathway is activated by water stress. Considering that the immediate epoxycarotenoid precursors are always present in large excess relative to ABA (Norman et al., 1990) and that the enzyme activities of the final two steps from xanthoxin to ABA are not affected by dehydration, it follows that the cleavage reaction from epoxycarotenoids to xanthoxin is the most likely regulatory step in the pathway.

But what is the nature of the enzyme that catalyzes the cleavage reaction? Recently, a viviparous mutant of maize, *vp14*, has been isolated, and the corresponding gene has been cloned. The derived amino acid sequence of VP14 shows similarity to bacterial dioxygenases. Moreover, a VP14 fusion protein catalyzes the cleavage of 9-*cis*-epoxycarotenoids (C_{40}) to form C_{25} apo-aldehydes and xanthoxin (C_{15}), but carotenoids in the all-*trans* configuration are not cleaved (Schwartz et al., 1997b). Therefore, the 9-*cis*-configuration appears to be the primary determinant of cleavage specificity. This is not surprising, because cleavage of 9-*cis*-epoxycarotenoids produces *cis*-xanthoxin, which is in turn converted to the active isomer of ABA.

The cleavage step is the first committed step in ABA biosynthesis and probably also the reaction that determines the overall rate of ABA production. This possibility can now be tested in transgenic plants in which the gene encoding the cleavage enzyme is over- or underexpressed. However, ABA is rapidly deactivated by oxidation to phaseic acid and, to a lesser extent, by conjugation to the ABA glucose ester (reviewed in Zeevaart and Creelman, 1988). Thus, overexpression of the

cleavage enzyme in transgenic plants may not necessarily result in increased ABA levels. To raise ABA levels and to make plants tolerant to stress conditions, it may be preferable to suppress the activity of ABA 8′-hydroxylase, the enzyme that converts ABA to phaseic acid, by using antisense technology. However, the experiments will have to wait until the corresponding gene has been cloned.

Ethylene

The breakthrough in unraveling the biosynthetic pathway of ethylene was the discovery in 1979 that 1-aminocyclopropane-1-carboxylic acid (ACC) is the immediate precursor of ethylene (reviewed in Yang and Hoffman, 1984). The first committed step in ethylene biosynthesis is the conversion of *S*-adenosyl-L-methionine to 5′-methylthioadenosine and ACC; this is also the key regulatory step in ethylene biosynthesis.

The enzyme that catalyzes this reaction, ACC synthase, was partially purified before the corresponding gene was cloned (reviewed in Kende, 1993; Zarembinski and Theologis, 1994). ACC synthase is encoded by a multigene family whose members are differentially expressed in response to developmental, environmental, and hormonal factors. For example, by using gene-specific probes, the differential expression of tomato ACC synthase family members has been investigated. Transcripts of one isoform increased during fruit ripening, those of another increased in response to wounding, and those of a third form increased in response to treatment with auxin (Olson et al., 1991; Yip et al., 1992). ACC synthase genes expressed in response to a particular stimulus (e.g., the application of auxin) are more similar to genes controlled by the same stimulus in other species than they are to other ACC genes in the same species (Liang et al., 1992; Trebitsh et al., 1997).

The final step in ethylene biosynthesis, the conversion of ACC to ethylene, is catalyzed by ACC oxidase. ACC oxidase was first identified by expressing the tomato cDNA pTOM13 in an antisense orientation, which resulted in greatly reduced ethylene production in tomato (Hamilton et al., 1990). The deduced amino acid sequence of pTOM13 is similar to that of dioxygenases that require Fe^{2+} and ascorbate as cofactors. When these cofactors were added to assays for ACC oxidase, enzyme activity was completely recovered (Ververidis and John, 1991). Later, it was found that CO_2 is also an essential activator of ACC oxidase (Fernández-Maculet et al., 1993).

Numerous cDNAs for ACC oxidase have been isolated from different species (see Barry et al., 1996). As is the case with ACC synthase, ACC oxidase is encoded by small multigene families. Although the initial evidence indicated that ethylene synthesis is controlled at the level of ACC synthase, there is now considerable evidence that ACC oxidase also plays a significant role in regulating ethylene biosynthesis. By using gene-specific probes for three ACC oxidase genes of tomato, distinct patterns of expression in various organs and at different stages of development have been observed (Barry et al., 1996). Moreover, the positive feedback loop in which treatment of tissue with ethylene often stimulates ethylene production by that tissue appears to take place through enhanced expression of ACC synthase and ACC oxidase (reviewed in Kende, 1993).

Besides being converted to ethylene, ACC can also be irreversibly conjugated to form *N*-malonyl-ACC (Kionka and Amrhein, 1984). Malonylation of ACC regulates the level of ACC and thus the production of ethylene. Ethylene can be metabolized by plant tissues to ethylene oxide and ethylene glycol (Sanders et al., 1989), but the physiological significance of this metabolism remains to be established. As a gas, ethylene can readily diffuse from plant tissues, so metabolism is not essential for its removal.

HORMONES AND VEGETATIVE GROWTH

Plant growth is based on the production of cells in the meristems and the ensuing elongation of these newly formed cells (see Clark, 1997; Cosgrove, 1997; Kerstetter and Hake, 1997; Schiefelbein et al., 1997, in this issue). As has been well documented, plant hormones affect both cell division and cell elongation. Here, we use selected examples to illustrate how auxin, cytokinin, and GA promote cell division.

Cell-Cycle Control

Auxin stimulates radish root pericycle cells arrested in the G2 phase of the cell cycle to enter into mitosis, thereby promoting the formation of lateral root primordia (Blakely and Evans, 1979). Similarly, the role of kinetin as the factor required for the maintenance of cell division activity in tissue cultures is well known (Miller et al., 1955). However, the role of endogenous cytokinins in controlling cell division in intact plants has not been demonstrated unequivocally. In part, this is because two tools that have been very valuable in determining the physiological functions of some plant hormones, namely, well-defined biosynthesis or perception mutants and chemical inhibitors of hormone action, are not available for cytokinins. GA induces stem growth in many rosette plants and dwarf mutants. This growth response can be quite dramatic and is the combined result of enhanced cell division activity in the subapical meristem and increased cell elongation. Sachs et al. (1959) provided a well-documented example of GA-promoted mitosis in the subapical meristem of the long-day plant *Samolus parviflorus.*

Despite these early indications that hormones regulate cell division and growth, two broad questions regarding hormonally stimulated cell division activity still require resolution: do plant hormones influence cell division directly or indirectly, and at what point of the cell cycle do plant hormones

act? Answers to the first question are mostly lacking; answers to the second are beginning to emerge.

In the intercalary meristem of deepwater rice, GA promotes cell division and cell elongation (Sauter and Kende, 1992). This leads to internodal growth rates of up to 5 mm/hr. Measurements of cell length and determinations of the progress of cells through the cell cycle by flow cytometry and ^{3}H-thymidine incorporation indicated that GA-induced cell elongation preceded the promotion of cell division. Therefore, it has been proposed that the primary action of GA in the intercalary meristem of rice is on cell elongation and that entry into the cell cycle is a function of cell size, a phenomenon that has been well documented in yeast (e.g., Nurse, 1991).

Passage of cells through phases of the mitotic cycle is controlled by a family of serine/threonine protein kinases and their regulatory subunits, the cyclins (see Jacobs, 1995, 1997, in this issue). The promotion of cell division activity by plant hormones is reflected in hormonally induced activities of cyclin-dependent p34^{cdc2}-like protein kinases, in the level of their mRNAs, and also in the level of cyclin transcripts.

For example, John et al. (1993) showed that auxin alone increased the level of a p34^{cdc2}-like protein in cultured tobacco cells, but addition of a cytokinin was required for activation of this kinase. Because cytokinin increased the abundance of cyclin mRNA in suspension-cultured Arabidopsis cells (Soni et al., 1995), it is conceivable that the observed activation of the p34^{cdc2}-like protein kinase in tobacco cells was based on the synthesis of a cyclin. Similarly, auxin has been reported to increase both p34^{cdc2} and cyclin mRNA levels in roots in conjunction with the induction of cell divisions (e.g., Miao et al., 1993; Ferreira et al., 1994). Moreover, GA promotes the activity of a p34^{cdc2}-like protein kinase and the expression of genes encoding a p34^{cdc}-like protein kinase and cyclin homologs in the intercalary meristem of deepwater rice (Sauter et al., 1995). However, in no case has it been shown that plant hormones regulate directly the expression of genes that code for regulatory proteins of the cell cycle.

A completely different aspect of the hormonal control of the cell division cycle has been described by Houssa et al. (1994). These authors observed that exogenous cytokinin reduced the size of chromosomal DNA replication units in the shoot meristems of *Sinapis alba* and *Lolium temulentum* and in ovules of tomato. On the basis of these data, Houssa et al. (1994) proposed that the activation of latent replication origins is a universal effect of cytokinins in the promotion of cell division.

Hormones and Cell Expansion

Auxin and GA are viewed as hormones that promote cell elongation. Growth of plant cells is driven by water uptake, which in turn results from stress relaxation of the cell wall (Cosgrove, 1993, 1997, in this issue). To promote growth, plant hormones are expected to cause loosening of the cell wall, but how is this achieved? The acid-growth theory postulates that secretion of hydrogen ions into the cell wall is stimulated by auxin and that the lowered pH in the apoplast activates wall-loosening processes (Rayle and Cleland, 1970; Hager et al., 1971). Indeed, there are several lines of evidence that support this hypothesis, in particular, the facts that auxin causes acidification of the cell wall and that acidic buffers induce growth in auxin-sensitive tissues (Rayle and Cleland, 1992).

However, the acid-growth theory of auxin action is not universally accepted. For example, critics point out that the wall pH in auxin-treated tissues is not low enough to elicit the growth rates observed. Nevertheless, all postulates of the acid-growth hypothesis have been shown to hold for growth induced by the fungal toxin fusicoccin (Kutschera, 1994). Technical difficulties, such as determining the pH of the cell wall, appear to have precluded a resolution of this controversy. The discovery of the expansins, a family of proteins that exhibit wall-loosening activity at pH ~4.5 (Cosgrove, 1996, 1997, in this issue), may open new approaches to the problem of hormonally induced stress relaxation of the cell wall. It will be necessary to establish the role of the expansins in growth by, for example, genetic manipulation of their expression and to examine the connection, if any, between hormone levels and expansin action.

The relationship between auxin and GA action also awaits resolution. It was thought that auxin-stimulated growth could only be observed in excised, auxin-depleted tissue, whereas GA could induce the growth of intact plants. However, exceptions to this generalization have recently been reported. For example, it has been shown that the continuous supply of auxin via a cotton wick stimulates growth of two dwarf mutants of pea, *le* and *lkb* (Yang et al., 1996). The first is a GA-deficient mutant whose growth can be fully restored by treatment with GA_1 (Ingram et al., 1983). The latter is a dwarf mutant with a two- to threefold reduction in IAA level (McKay et al., 1994) whose growth is not promoted by applied GA (Yang et al., 1996). On the basis of these data, it was concluded that auxin and GA control separate processes in stem elongation. GA may act preferentially in younger cells compared with auxin and induce cell division and cell elongation, whereas auxin may act by promoting cell extension (Yang et al., 1996).

How could these observations be explained? For a cell to elongate, its load-bearing cellulose microfibrils must be oriented perpendicular to the direction of growth (Green, 1980). Induction of cell elongation by GA may be confined to meristematic and young cells because their cellulose microfibrils are orientated transversely. Under the influence of GA, this transverse orientation of the cellulose microfibrils is maintained over a longer distance, thus extending the elongation zone of the organ (Sauter et al., 1993). By contrast, auxin is known to cause a reorientation of cellulose microfibril deposition from the oblique/longitudinal to the transverse and thereby promote the elongation of cells that have

stopped growing (Bergfeld et al., 1988). This may explain why GA action usually requires the presence of a meristem, where GA promotes cell elongation and, perhaps indirectly, cell division (Sauter and Kende, 1992), whereas auxin can promote elongation of older cells in the absence of a meristem (see Jacobs, 1997, in this issue).

Ethylene inhibits the elongation of terrestrial plants and causes thickening of their stems. This effect has been ascribed to a reorientation of both the cortical microtubules and the newly deposited cellulose microfibrils from mostly transverse to mostly oblique/longitudinal (Lang et al., 1982; Roberts et al., 1985). By contrast, the rapid elongation of many semiaquatic plants upon submergence is mediated by ethylene, which accumulates in the submerged tissue (Voesenek et al., 1992). It has been shown for two semiaquatic plants, *Callitriche platycarpa* (Musgrave et al., 1972) and deepwater rice (Raskin and Kende, 1984), that ethylene acts by increasing the tissue's responsiveness to GA and that GA is the immediate growth-promoting hormone.

Further support for this hypothesis comes from experiments with deepwater rice in which ethylene treatment led to a rapid decline of endogenous ABA levels (Hoffmann-Benning and Kende, 1992). Because ABA is a potent antagonist of GA action in rice, it has been suggested that the increased responsiveness to GA is based on reduced ABA content. In other words, the growth rate of the plant would be determined by the balance of an inhibitor (i.e., ABA) and a promoter (i.e., GA) of growth. In support of this hypothesis, it was shown that elongation of rice coleoptiles, whose growth is also promoted by ethylene, is stimulated by fluridone, an inhibitor of carotenoid and ABA biosynthesis (Hoffmann-Benning and Kende, 1992).

HORMONAL TRANSDUCTION PATHWAYS IN GROWTH

The first attempts to identify plant hormone receptors were based on hormone binding experiments. More recently, isolation of Arabidopsis hormone response mutants led to the identification of putative hormone receptors and elements of hormonal transduction pathways. In some instances, these elements fit to existing paradigms of signal transduction pathways. In other instances, they do not, or the function of the respective gene products is still unknown. We limit our discussion to components of hormonal transduction pathways whose protein sequences have been elucidated and for which some function can be suggested.

Identification of the first putative hormone receptor in plants, an auxin binding protein (now called ABP1), was based on the binding experiments of Hertel et al. (1972); purification of the protein and cloning of the corresponding cDNA showed the presence of an endoplasmic reticulum localization signal (ABP1 and other auxin binding proteins are reviewed in Jones, 1994). An ABP1 homolog or an immunologically related protein is also localized at the plasma membrane. Evidence for a receptor function of the plasma membrane–bound ABP comes from experiments showing that auxin-induced hyperpolarization is inhibited by antibodies against ABP1 (Barbier-Brygoo et al., 1989). It is not known, however, whether ABP1 plays any role in mediating growth.

A number of "auxin-resistant" mutants of Arabidopsis have been isolated for the genetic dissection of the auxin transduction pathway (Walden and Lubenow, 1996). These mutants were selected for their ability to grow on high concentrations of auxin. By using this strategy, Leyser et al. (1993) showed that the product of one such auxin resistance gene, *AXR1*, has similarity to the ubiquitin-activating enzyme E1. Mutants in the *AUX1* gene of Arabidopsis show auxin resistance with respect to root growth and do not exhibit root gravitropism. The product of the *AUX1* gene has sequence similarities to plant and fungal amino acid permeases, indicating a role for this protein in auxin transport (Bennett et al., 1996).

Several genes rapidly upregulated by auxin application have been identified (Abel and Theologis, 1996), of which two classes are described briefly. McClure et al. (1989) characterized the so-called small auxin up RNA (*SAUR*) genes from soybean, some of which are auxin regulated. When soybean seedlings were oriented horizontally, expression of *SAUR* genes became asymmetrical, that is, the level of *SAUR* mRNA on the lower side of the hypocotyl was much higher than on the upper side (McClure and Guilfoyle, 1989). This may indicate that asymmetrical expression of *SAUR* genes results from lateral redistribution of auxin in gravitropically stimulated stems, as postulated by the Cholodny-Went hypothesis. Another family of early auxin-induced genes has been described by Abel et al. (1994). They encode short-lived nuclear proteins that may act as activators or repressors of genes whose products mediate auxin responses.

Progress has also been made in identifying components of the signal transduction pathway in GA-regulated growth by using Arabidopsis response mutants (Swain and Olszewski, 1996). Such mutants fall into two categories: those that have a dwarf phenotype but do not grow in response to GA, and those that grow tall and slender, mimicking the effects of GA treatment. The *spindly* (*spy*) mutant falls into the latter category and is defective in a tetratricopeptide repeat–containing protein that may be involved in protein–protein interactions (Jacobsen et al., 1996). A similar approach led to the identification of the Arabidopsis gene *ABA-INSENSITIVE1* (*ABI1*) (Leung et al., 1994; Meyer et al., 1994). A mutation in this gene renders plants insensitive to ABA. *ABI1* was found to encode a protein with high similarity to a 2C-class serine/threonine protein phosphatase with an N-terminal calcium binding site. This indicates that the ABI1 protein functions as a calcium-regulated protein phosphatase and is thus part of a phosphorylation-dependent transduction pathway that mediates a broad spectrum of ABA responses.

Substantial progress has been made in elucidating the ethylene transduction pathway by screening for ethylene response mutants in Arabidopsis (Ecker, 1995). In the pres-

ence of ethylene, dark-grown seedlings of such mutants either do not exhibit the "triple response" (i.e., stunted growth, swelling of the root and hypocotyl, and exaggerated apical hook formation) or show the triple response phenotype even in the absence of ethylene. We limit our discussion of ethylene signal transduction to four components of the pathway whose functions have been derived from sequence analysis of the corresponding genes and from direct ethylene binding experiments.

The mutation *ethylene-resistant1* (*etr1*) is dominant, and the mutant lacks a number of responses to ethylene, including inhibition of cell elongation, promotion of seed germination, enhancement of peroxidase activity, acceleration of leaf senescence, and feedback inhibition of ethylene biosynthesis (Bleecker et al., 1988). The capacity of *etr1* to bind ethylene in vivo was one-fifth that of the wild type, indicating that the mutant is impaired in receptor function.

The *ETR1* gene was isolated by map-based cloning and was found to encode a protein with sequence similarity to bacterial two-component regulators (Chang et al., 1993). In its N-terminal portion, it contains a sensor domain with a putative input and histidine kinase region (Figure 3); fused to this is a receiver domain, but the equivalent of an output domain is missing. Using protein expressed in yeast, Schaller and Bleecker (1995) showed that the hydrophobic N-terminal region of ETR1 binds ethylene and that the *etr1-1* mutation, which is localized in this region and which leads to ethylene insensitivity, abolishes ethylene binding. These results constitute compelling evidence that ETR1 is an ethylene receptor and that the ethylene binding site is located in a membrane-spanning region of the N-terminal input domain. The *ETR2* and *ETHYLENE-INSENSITIVE4* (*EIN4*) genes encode homologs of *ETR1*, and mutations in these genes confer dominant ethylene insensitivity onto Arabidopsis seedlings (Roman et al., 1995; Hua et al., 1997).

Hua et al. (1995) have cloned an Arabidopsis gene, *ETHYLENE RESPONSE SENSOR* (*ERS*), that encodes a second type of putative ethylene receptor. The sensor domain of the ERS protein shows high similarity to ETR1, but it lacks a receiver domain (Figure 3). When, by site-directed mutagenesis, the same amino acid change was introduced into ERS, as was found in the mutant protein ETR1-4, the corresponding transgenic plants showed dominant ethylene insensitivity. Thus, Arabidopsis contains at least four genes that encode putative ethylene receptors, *ETR1*, *ETR2*, *EIN4*, and *ERS*. This potential redundancy could explain why no loss-of-function mutations have been found for any of these genes. Redundancy in ethylene perception is also evident from the fact that intragenic suppressor mutations in *etr1* resulted in recovery of ethylene sensitivity (Hua et al., 1997). Homologs of *ETR1* and *ERS* have also been isolated from tomato (Wilkinson et al., 1995; Zhou et al., 1996).

Genes acting downstream of ethylene reception in Arabidopsis include *CONSTITUTIVE TRIPLE RESPONSE* (*CTR1*). *ctr1* mutants express the triple-response phenotype constitutively, even in the absence of ethylene (Kieber et al., 1993). Genetic analyses have shown that *CTR1* acts downstream of *ETR1*, *ETR2*, *EIN4*, and *ERS* and that it is a negative regulator of ethylene responses. *CTR1* encodes a putative serine/threonine protein kinase that is related to Raf protein kinases. This relationship indicates that the ethylene transduction pathway may be similar to a mitogen-activated protein

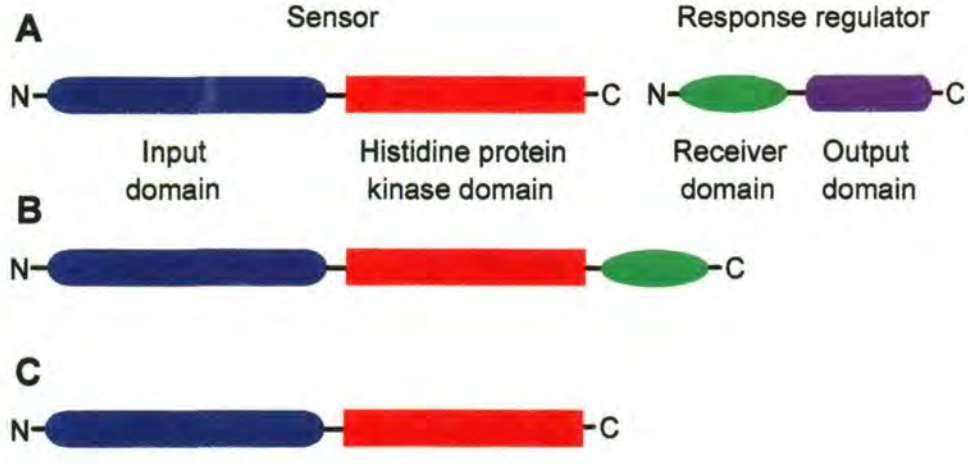

Figure 3. Two-Component Signaling Systems.

(A) The bacterial two-component system composed of sensor and response regulator proteins.

(B) The ETR-type two-component system. The ethylene binding site is located in the *trans*-membrane region of the input domain. The tomato eTAE1 homolog implicated in ethylene signal transduction (Zhou et al., 1996) and the CKI1 homolog implicated in cytokinin signaling (Kakimoto, 1996) are of this type.

(C) The ERS-type protein. ERS lacks the receiver domain; the tomato NR protein is a homolog of ERS (Wilkinson et al., 1995).

kinase–mediated phosphorylation cascade, albeit one linked to a receptor related to those typically associated with prokaryotic sensing systems.

A second downstream gene is *HOOKLESS1* (*HLS1*) of Arabidopsis, which was identified as an ethylene-responsive gene whose expression is required for the formation of the apical hook (Lehman et al., 1996). It has been suggested that the *N*-acetyltransferase encoded by *HLS1* affects the distribution of auxin in seedlings and as such could constitute a link between ethylene and auxin action in asymmetric growth.

Arabidopsis mutants that form calli and shoots in tissue culture without added cytokinin were isolated by activation T-DNA tagging (Kakimoto, 1996). The gene *CYTOKININ-INDEPENDENT1* (*CKI1*), which was tagged in four of these mutants, was found to encode a protein similar to two-component regulators. It has a putative histidine kinase and receiver domain and resembles ETR1. Thus, in all likelihood, it functions in the transduction of cytokinin responses and may indeed be a cytokinin receptor.

CONCLUSIONS AND PROSPECTS

Although combining the disciplines of biochemistry, molecular genetics, and physiology has led to major advances in our understanding of the role of hormones in plants, much more remains to be learned. The biosynthesis of IAA and cytokinins in higher plants is still poorly understood, and only in the case of ethylene has a receptor been identified. As the entire genome of Arabidopsis is sequenced over the next few years, a plethora of genes, including those involved in hormone metabolism and signal transduction, will become available; the challenge will be to determine their functions.

The levels of IAA and cytokinin have been altered in transgenic plants, mostly with constitutively expressed promoters (reviewed in Klee and Romano, 1994). Similar experiments with ABA and GA biosynthetic genes can be anticipated. In the future, these analyses should be refined by using specific promoters so that manipulation of hormone levels in certain cells, organs, and tissues and at specific times can be achieved (e.g., Gan and Amasino, 1995). Transgenic plants with modified hormone levels or altered hormone responses may offer an alternative to the practice of spraying plants with hormones to manipulate their growth and development.

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Indole-3-butyric acid in plant growth and development

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Key words: Arabidopsis thaliana, arbuscular mycorrhiza, biosynthesis, indole-3-acetic acid, indole-3-butyric acid, regulation, *Zea mays*

Abstract

Within the last ten years it has been established by GC-MS that indole-3-butyric acid (IBA) is an endogenous compound in a variety of plant species. When applied exogenously, IBA has a variety of different effects on plant growth and development, but the compound is still mainly used for the induction of adventitious roots. Using molecular techniques, several genes have been isolated that are induced during adventitious root formation by IBA. The biosynthesis of IBA in maize (*Zea mays* L.) involves IAA as the direct precursor. Microsomal membranes from maize are able to convert IAA to IBA using ATP and acetyl-CoA as cofactors. The enzyme catalyzing this reaction was characterized from maize seedlings and partially purified. The *in vitro* biosynthesis of IBA seems to be regulated by several external and internal factors: i) Microsomal membranes from light-grown maize seedlings directly synthesize IBA, whereas microsomal membranes from dark-grown maize plants release an as yet unknown reaction product, which is converted to IBA in a second step. ii) Drought and osmotic stress increase the biosynthesis of IBA maybe *via* the increase of endogenous ABA, because application of ABA also results in elevated levels of IBA. iii) IBA synthesis is specifically increased by herbicides of the sethoxydim group. iv) IBA and IBA synthesizing activity are enhanced during the colonization of maize roots with the mycorrhizal fungus *Glomus intraradices*. The role of IBA for certain developmental processes in plants is discussed and some arguments presented that IBA is *per se* an auxin and does not act via the conversion to IAA.

Abbreviations: ACCase – acetyl-CoA carboxylase; AM – arbuscular mycorrhiza; 4-Cl-IAA – 4-chloro-IAA; IBA – indole-3-butyric acid; PAA – phenylacetic acid; TFIBA – 4,4,4-trifluoro-3-(indole-3-)butyric acid

1. Introduction

Auxins are a class of phytohormones which are involved in many aspects of growth and development of plants [20]. The auxin indole-3-acetic acid (IAA) was the first plant hormone to be used to stimulate rooting of cuttings [17]. At this time it was discovered that a new, 'synthetic' auxin indole-3-butyric acid (IBA) also promoted rooting and was even more effective than IAA [99]. IBA is now used commercially world wide to root many plant species [35]. Since its introduction more than 50 years ago, IBA has been the subject of many experiments mostly involving trial and error studies of different concentrations, formulations, additives and treatment durations to achieve optimum rooting for the plant species in question. Today one can still find varieties and cultivars in almost every species that do not respond with rooting to different treatments with IBA. Many investigations have shown that it has a greater ability to promote adventitious root formation as compared with IAA, but this effect has been assumed to be due to the higher stability of IBA versus IAA in solution and in the tissue [71]. Although IBA is used in many laboratories, its role *in vivo* is still not clear. The elucidation of how IBA is made within the plant is one step towards understanding how it may function in plant development. Recent investigations on IBA bio-

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RESEARCH PAPER

Analysis of indole-3-butyric acid-induced adventitious root formation on *Arabidopsis* stem segments

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Abstract

Root induction by auxins is still not well understood at the molecular level. In this study a system has been devised which distinguishes between the two active auxins indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA). IBA, but not IAA, efficiently induced adventitious rooting in *Arabidopsis* stem segments at a concentration of 10 μM. In wild-type plants, roots formed exclusively out of calli at the basal end of the segments. Root formation was inhibited by 10 μM 3,4,5-triiodobenzoic acid (TIBA), an inhibitor of polar auxin transport. At intermediate IBA concentrations (3–10 μM), root induction was less efficient in *trp1*, a tryptophan auxotroph of *Arabidopsis* with a bushy phenotype but no demonstrable reduction in IAA levels. By contrast, two mutants of *Arabidopsis* with measurably higher levels of IAA (*trp2*, *amt1*) show root induction characteristics very similar to the wild type. Using differential display, transcripts specific to the rooting process were identified by devising a protocol that distinguished between callus production only and callus production followed by root initiation. One fragment was identical to the sequence of a putative regulatory subunit B of protein phosphatase 2A. It is suggested that adventitious rooting in *Arabidopsis* stem segments is due to an interaction between endogenous IAA and exogenous IBA. In stem explants, residual endogenous IAA is transported to the basal end of each segment, thereby inducing root formation. In stem segments in which the polar auxin transport is inhibited by TIBA, root formation does not occur.

Key words: Adventitious root formation, *Arabidopsis*, auxin, auxin-inducible proteins, differential display, indole-3-butyric acid, protein phosphatase 2A, TIBA.

Introduction

Root development in *Arabidopsis thaliana* has been the subject of many studies employing mutant screens during the last few years (for a review see Casson and Lindsey, 2003). While development of the primary root from the embryonic stage has received a lot of attention and the processes involved are beginning to unravel, the formation of lateral and adventitious roots is less well understood. Lateral and adventitious roots are formed post-embryonically. While lateral roots typically form from the root pericycle, adventitious roots form naturally from stem tissue. Adventitious roots are less predictable in their cellular site of origin than lateral roots. They may form from the cambium or, in the case of detached stem cuttings, from calli. Therefore it appears that adventitious roots can be formed by two different pathways: (i) direct organogenesis from established cell types or (ii) from callus tissue following mechanical damage (Casson and Lindsey, 2003, and references therein).

Adventitious root formation has many practical implications in horticulture and agronomy and there is a lot of commercial interest because of the many plant species that are difficult to root. (Davies *et al*., 1994; Kovar and Kuchenbuch, 1994). The auxin indole-3-acetic acid (IAA) was the first plant hormone to be used to stimulate rooting of cuttings (Cooper, 1935). At that time it was discovered

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that a second, 'synthetic' auxin indole-3-butyric acid (IBA) also promoted rooting and was even more effective than IAA (Zimmerman and Wilcoxon, 1935). IBA is now used commercially worldwide to root many plant species (Hartmann *et al*., 1990). Since its introduction more than 50 years ago, IBA has been the subject of many experiments, mostly involving trial and error studies to achieve optimum rooting conditions for the plant species in question. Application of IBA to cuttings of many plant species results in the induction of adventitious roots, in many cases more efficiently than IAA (Epstein and Ludwig-Müller, 1993). For example, in *Vigna radiata* the induction of adventitious roots was observed after IBA, but not IAA application (Riov and Yang, 1989). The greater ability of IBA to promote adventitious root formation compared with IAA has been attributed to the higher stability of IBA versus IAA both in solution and in plant tissue (Nordström *et al*., 1991). The effective concentration of IBA in these kinds of studies was also dependent on the pH of the medium. It was shown that, at lower pH values, lower IBA concentrations in the medium were sufficient to induce rooting of apple cuttings (Harbage and Stimart, 1996).

Differences in the ability to form adventitious roots have been attributed to differences in auxin metabolism (Alvarez *et al*., 1989; Blazkova *et al*., 1997; Epstein and Ludwig-Müller, 1993). It was shown, for example, that a difficult-to-root cultivar of *Prunus avium* conjugated IBA more rapidly than an easy-to-root cultivar (Epstein *et al*., 1993). Only in the easy-to-root cultivar was the appearance of free IBA observed after several days and the authors concluded that the difficult-to-root cultivar was not able to hydrolyse IBA conjugates during the appropriate time points of adventitious root development. Interestingly, it was possible to induce rooting of the difficult-to-root cultivar after application of an inhibitor of conjugation (Epstein *et al*., 1993). It has been shown that IBAsp is even more active than free IBA in the promotion of adventitious roots in mung bean, possibly due to its higher stability during the rooting process (Wiesman *et al*., 1989). However, other differences such as uptake and transport can also account for the differences in rooting behaviour (Epstein and Ludwig-Müller, 1993).

The physiological events leading to root initiation may be revealed by using targeted or untargeted molecular approaches to identify genes that may be involved in adventitious rooting. IBA has been identified as a natural substance in *Arabidopsis thaliana* (Ludwig-Müller *et al*., 1993) and there are indications that at least part of the action of IBA is not through IAA in this species (Poupart and Waddell, 2000; Zolman *et al*., 2000). Therefore a system has been devised for adventitious root formation on stems of the model plant *Arabidopsis* under sterile conditions, where roots are specifically induced after the application of IBA but not of IAA. The results have shown that (i) IBA is one important factor in *Arabidopsis* to induce adventitious roots, (ii) the timing of auxin application is important to distinguish between callus and root formation, and (iii) this system is suitable for identifying genes involved in adventitious root formation. Finally, the effect of an auxin transport inhibitor, TIBA, on IBA-induced adventitious root formation has been investigated and IAA-deficient mutants were used to analyse the interplay between IAA and IBA during adventitious rooting.

Materials and methods

Plant material

Arabidopsis plants were grown aseptically on Murashige and Skoog (MS) agar (Murashige and Skoog, 1962) in Magenta® boxes at 24 °C under constant illumination with cool-white fluorescent lights, approximately 40 μmol m^{-2}. The seeds were surface-sterilized with 5% (v/v) commercial bleach (Clorox; a 5% solution of sodium hypoclorite) for 20 min, washed thoroughly, planted on 1% agar, and vernalized for 24 h at 4 °C. Inflorescences from 4–8-week-old-plants were used because, during this period, the age of the stems did not influence callus/root formation, although on stem segments of older plants no root formation could be observed; data not shown). The inflorescences were cut into 0.5 cm node-free segments and incubated in the dark or under constant illumination in Petri dishes containing full-strength MS agar containing the appropriate concentrations of IAA or IBA with or without different concentrations of 3,4,5-triiodobenzoic acid (TIBA). In the light, the plates were covered with yellow plastic to prevent photo-oxidation of auxins (Campanella *et al*., 1996). Starting at 5 d, plates were examined daily and the proportion of segments showing callus or root formation was scored.

For the differential display experiments, segment length was reduced to 3 mm to increase the number of ends per fresh weight. For the subsequent treatments, segments were transferred under sterile conditions to fresh Petri dishes containing either plain MS agar or MS agar with the appropriate hormone supplement.

For histology, stem segments were fixed for at least 24 h in FAA (5% formaldehyde, 5% acetic acid, 50% ethanol), then dehydrated through a series of ethanol steps (70%, 80%, 95%) before infiltration with JB-4 resin (Polysciences, Inc., Niles, IL). Sections of 2–4 μm were stained with toluidine blue.

Evaluation of the rooting process

On each Petri dish for the different treatments 10–12 *Arabidopsis* stem segments were placed. Each experimental condition consisted of at least two Petri dishes. All experiments were performed at least three times, resulting in a minimum of 60 segments which were scored per treatment. Mean values of the three independent experiments are given. After the different treatments the *Arabidopsis* stem segments were inspected for callus or root formation and the number of segments exhibiting the respective organs counted.

RNA extraction and differential display

Isolation of total RNA was performed using TRIzol reagent (Gibco BRL, now marketed by Invitrogen, Carlsbad, CA) according to the manufacturer's instructions using 300 mg fresh weight of treated and control segments. Reverse transcription followed by PCR using anchored VNT_{11} 3′-primers and 10-mer OPA 5′-primers (both Operon Technologies) was performed essentially as described by Liang and Pardee (1992). ^{35}S-Radiolabelled amplification products were resolved on 6% acrylamide sequencing gels and detected by autoradiography. The experiment was repeated to show reproducibility of fragment induction. Fragments induced only under condition C

were excised, re-amplified with the same primer combination, the PCR products purified (QIAquick® gel extraction kit, Qiagen), ligated into pBSK vector, and sequenced from both ends at The Institute for Genomic Research.

Northern blot analysis

Total RNA was isolated as described above. The synthesis of the biotinylated (bio-dUTP, Boehringer Mannheim) cDNA probe used for northern hybridization was performed by PCR. Template was cDNA prepared from total RNA of *Arabidopsis* stems induced with IBA. For amplification of the phosphatase 2A-like protein subunit as a probe, the following primer pair was designed according to the sequence information obtained: forward 5′-GATCATGTGATA-GAAGATAAATTTAGTGCT-3′; reverse 5′-TCTTCTATCACAT-GATCTCGTCAGGGACCA-3′. PCR was performed according to standard procedures using the following programme: initial denaturation at 96 °C for 5 min, followed by 30 cycles of 96 °C for 45 s, 55 °C for 45 s, 72 °C for 45 s. Equal sample loading (20 µg total RNA) was confirmed by hybridization with an *actin 2* (At3g18780) probe amplified with the following primers: forward 5′-GAAGAT-TAAGGTCGTTGCACCACCTG-3′; reverse 5′-ATTAACATTG-CAAAGAGTTTCAAGGT-3′. Non-radioactive northern blots were performed according to Löw and Rausch (1994), with the Northern-Light™-kit from Tropix (Serva) for detection.

Results

Indole-3-butyric acid can induce adventitious roots on Arabidopsis *stem segments*

Several reports deal with the better performance of IBA versus IAA during the rooting process. This was attributed to parameters such as stability, transport, or metabolism. Therefore a protocol was devised which would induce adventitious roots on *Arabidopsis* stems by one of the auxins but not the other. This study's experiments showed that several parameters influenced adventitious root induction and helped to discriminate between the actions of IAA and IBA. These were: (i) concentration of the hormone, (ii) duration of treatment, (iii) priming event, and (iv) second hormone treatment.

In a first set of experiments, 0.5 cm explants of *Arabidopsis* stems were incubated for 7 d on MS medium containing either IAA or IBA at different concentrations and the phenotype was recorded (Fig. 1A). Since the explants looked similar when they were cultivated on hormone plates for 7 d, only the explants on different IBA concentrations are shown. The induction of adventitious roots was always preceded by callus formation. Root induction was seen at 1 µM and 10 µM IBA and IAA, and at 100 µM hormone the roots looked stunted with more root hairs produced (Fig. 1A). Similarly, root induction by IAA or IBA was also possible using excised leaves (Fig. 1B). The concentration dependence was also comparable with that for stem segments.

On stem segments treated with IBA, adventitious roots clearly arose from the cambium, which first de-differentiates to form a callus (Fig. 2B). This is followed by the formation of roots (Fig. 2C) that subsequently elongated and by the formation of additional callus areas, which gave rise to new adventitious roots (Fig. 2D). In the controls without IBA such structures were never visible (Fig. 2A).

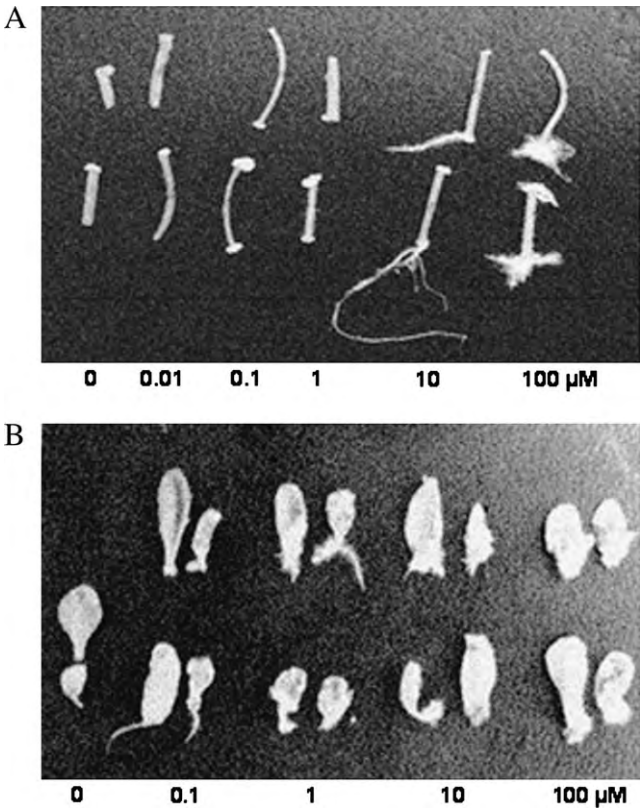

Fig. 1. Root induction on *Arabidopsis* stem (A) and leaf (B) explants after treatment with different IBA concentrations.

Timing of hormone requirement for adventitious rooting

To determine the period of IBA exposure required for adventitious root induction, the stem segments were incubated on 10 µM IAA or IBA for different time periods up to 48 h and then transferred to hormone-free MS medium for the remaining time. Callus and root formation was scored at 7 d (Fig. 3). The proportion of explants forming callus increased up to 100% after 48 h on auxin-containing medium (Fig. 3A). While callus formation was comparable on IAA- or IBA-containing MS agar, root formation was found only when IBA was in the medium. After a 6 h exposure, a response was already found, but optimum rooting was observed with a treatment of 48 h (Fig. 3B). After longer incubation periods the difference between IAA and IBA treatment became less pronounced (data not shown). The inset in Fig. 3B shows a picture of stem segments incubated for the respective time on either 10 µM IAA or 10 µM IBA.

A two-stage treatment was developed to distinguish between callus and root induction by IBA (Fig. 4). In stage I,

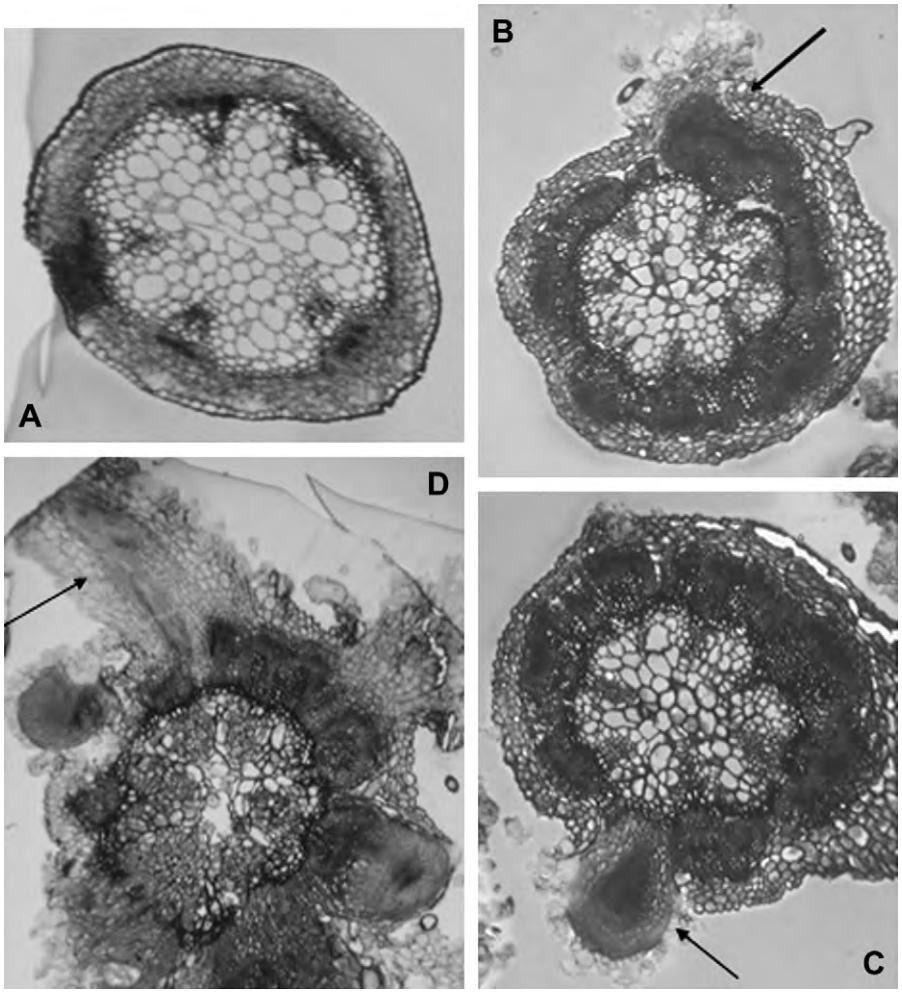

Fig. 2. Development of adventitious roots on *Arabidopsis* stems without (A) and after treatment with 30 μM IBA (B–D). Sections were taken 3 d (B), 5 d (C), and 9 d (D) after placing the segments on rooting medium. Sections of 2–4 μm were stained with toluidine blue. Adventitious roots are marked by arrows.

explants were incubated for 24 h on 10 μM IBA, a treatment that resulted in callus formation. In stage III, explants were given a second 10 μM IBA treatment of variable duration after a period of 24 h on hormone-free medium (stage II). The explants were transferred to hormone-free medium after the second IBA treatment for the remainder of the experiment (stage IV) and root formation was scored 14 d after the start of the second treatment. The second treatment resulted in the formation of adventitious roots on 60–95% of the explants, provided that it was at least 48 h long (Figs 4A, 5A). In addition, it was shown that the highest rooting efficiency was found with treatments that involved two exposures to IBA separated by a time without hormone (Fig. 5A). Increasing the incubation time of the second treatment on IBA also resulted in more segments showing adventitious root formation. Interestingly, in the experiments using only one long IBA treatment (Fig. 5B–D), more roots were formed when the treatment started with MS medium alone.

The auxin concentration was also important for the second treatment in which the explants were incubated for 48 h with different concentrations of IBA. Again with 1 μM and 10 μM IBA good induction of adventitious rooting was found with up to 95% of the segments showing roots (Fig. 5B). Callus formation without subsequent root formation was observed at concentrations <0.1 μM IBA.

Identification of transcripts expressed during adventitious rooting using differential display

The treatments of *Arabidopsis* stems described above were used to test this system for its suitability to isolate differentially expressed genes during adventitious rooting. Since the experimental procedure allowed the difference between callus formation and adventitious rooting to be distinguished, the comparison of control stems with stems treated to form callus or adventitious roots should provide transcripts which are specific for the rooting process. The

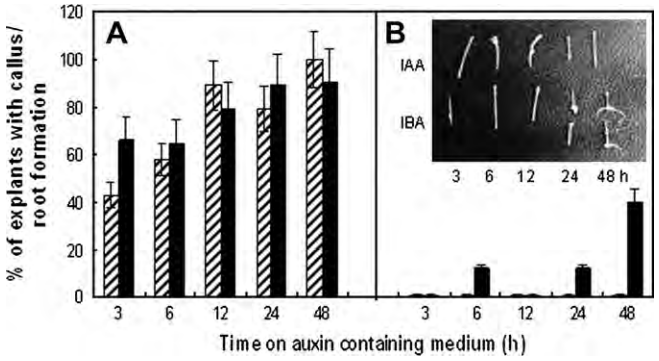

Fig. 3. Callus (A) and root (B) formation after continuous treatment with 10 μM IAA (hatched bars) or IBA (black bars) for different times on MS medium. The photograph shows the phenotype of rooted stem segments incubated for different periods on 10 μM IAA or IBA.

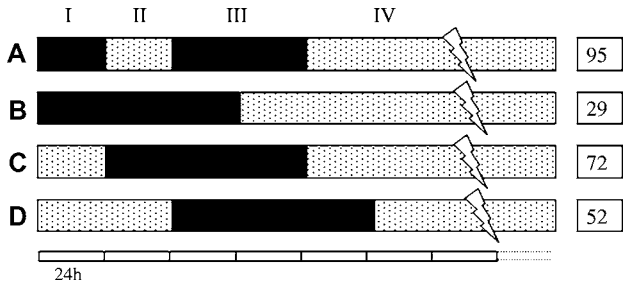

Fig. 4. Adventitious root formation is increased by a two-stage treatment and does not require continuous exposure to IBA. Different treatments with IBA are marked as follows: (dotted section) MS only, (black section) MS+10 μM IBA. The different variations tested are: (A) 24 h IBA/24 h MS/48 h IBA/MS; (B) 72 h IBA/MS; (C) 24 h MS/72 h IBA/MS; (D) 48 h MS/72 h IBA/MS. The segments were placed either on MS medium or MS supplemented with IBA after the indicated time periods (see time scale; one white bar segment represents 24 h). Percentage of root formation under the respective treatment conditions is given to the right of the bar. The stages mentioned in the text are indicated above the respective bar in Roman numerals. I: first IBA treatment; II: first period on MS; III: second IBA treatment; IV: remaining time until roots are visible on MS). The flash indicates a discontinuous time scale.

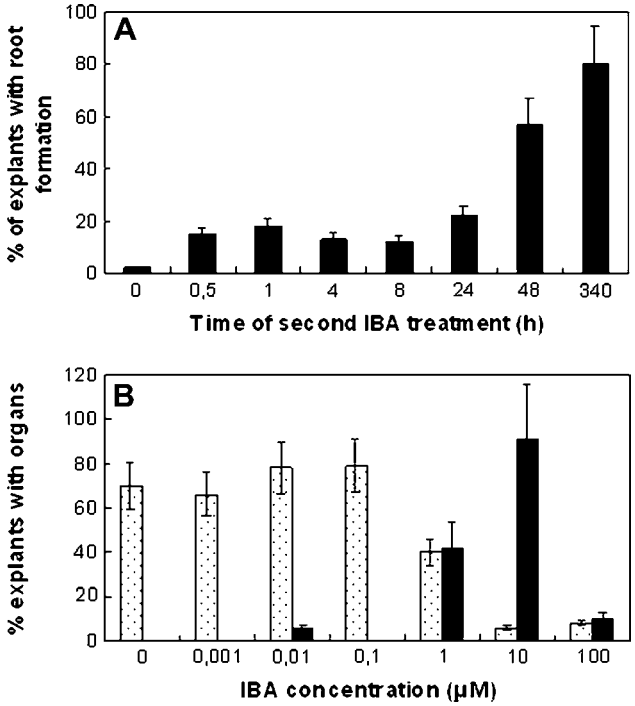

Fig. 5. Two-stage treatment for distinction between callus (dotted bars) and root (black bars) formation. (A) Dependence of root formation on the duration of the second 10 μM IBA treatment. Segments were transferred from IBA-containing medium to medium without hormone after the respective time periods. (B) Optimum concentration of IBA for the second treatment (340 h). The medium for the second treatment was supplemented with different IBA concentrations.

following three tissue samples were compared: (i) untreated segments; (ii) segments exposed to 10 μM IBA for 24 h, which will induce callus but not root formation; and (iii) segments given 24 h 10 μM IBA, 24 h no IBA, 48 h 10 μM IBA (see Fig. 4, Regime A), which induces roots in a large fraction of the explants (Fig. 6A). For the differential display experiment, a set of arbitrary primers (OPA1-12) was used in combination with anchor primers on each of the three mRNA populations described above. Bands specific to treatment C (root induction) were obtained with OPA primers 1, 6, and 12 (data not shown). Fragments designated 01-a, 01-b, 06-a, and 12-a were excised, reamplified and further analysed. It was not possible to reamplify fragment 12-a, therefore only three differentially expressed fragments remained. In all three cases only short fragments were amplified from the 3′-end. Therefore, all sequences are 3′-UTRs of the respective cDNAs. Since the completion of the *Arabidopsis* genome sequencing project, identification of gene sequences has been much facilitated. One 390 bp fragment (01-a) was homologous to a regulatory subunit B of protein phosphatase 2A (At3g54930). A second 340 bp fragment (01-b) was found to be derived from At1g29470 which was annotated as similar to the early-responsive dehydration stress protein, ERD3 that contains a putative methyltransferase motif. A third 300 bp fragment (06-a) was derived from At5g48545, a gene encoding an unknown protein of the histidine triad family protein with a HIT domain (http://www.tigr.org/tdb/e2k1/ath1/). Expression analysis confirmed the presence of the PP2A homologous mRNA specifically in tissues after IBA-induced adventitious root formation (Fig. 6B).

The polar auxin transport inhibitor TIBA inhibits adventitious root formation

Factors important for the effect of auxins during rooting might be (i) synthesis, (ii) metabolism, and (iii) transport. The latter was tested by using the polar auxin transport inhibitor 3,4,5-triiodobenzoic acid (TIBA) concomitantly with the IBA treatment leading to adventitious roots. Inhibition of root formation was observed when varying

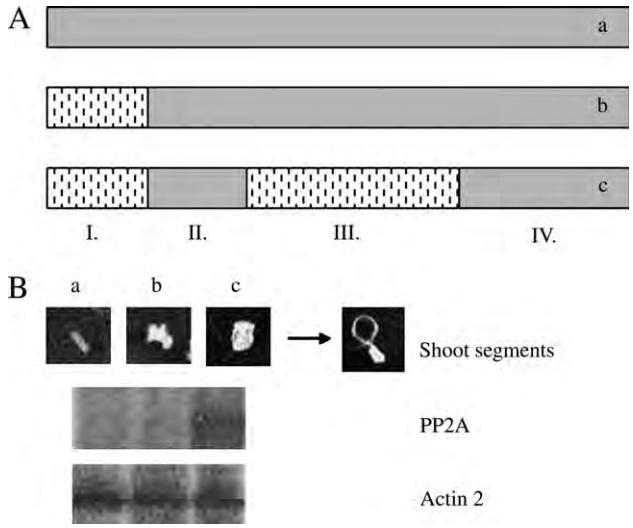

Fig. 6. Identification of transcripts specifically expressed during adventitious root formation by IBA. (A) The different treatment of segments is shown: (a) untreated segments; (b) segments exposed to 10 μM IBA for 24 h, which will induce callus but not root formation; (c) segments given 24 h 10 μM IBA, 24 h no IBA, 48 h 10 μM IBA. (dashed section) Time on IBA, (grey section) MS only. (B) RNA expression analysis of one fragment (PP2A B-regulatory subunit) in the three different tissues. The small photographs (a–c) show the segments as they looked after the harvest. Root development in treatment (c) was only observed later (indicated by an arrow). The Roman numerals correspond to those in Fig. 4.

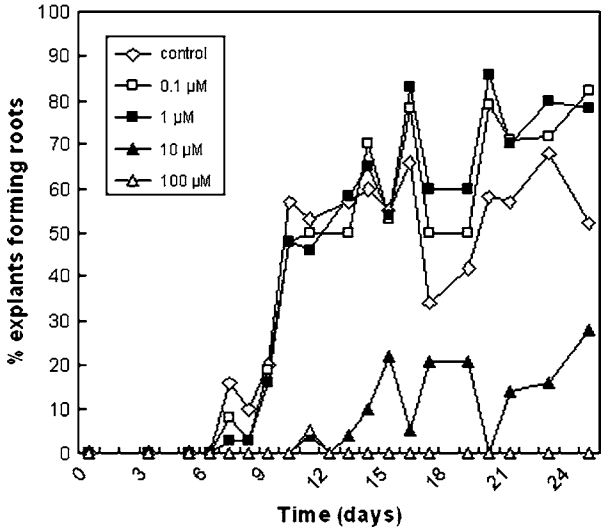

Fig. 7. Adventitious root formation at different concentrations of the IAA transport inhibitor TIBA in wild-type plants over a time period of 4 weeks. IBA was always at 10 μM. Inhibition of adventitious root formation was found at equimolar concentrations of IBA and TIBA.

concentrations of TIBA were added together with a fixed concentration of IBA (10 μM) in the medium (Fig. 7). While 0.1 μM and 1 μM TIBA had no inhibitory effect, 10 μM TIBA was already inhibitory and 100 μM TIBA completely prevented adventitious root formation. With lower TIBA concentrations there even seemed to be a small promoting effect after longer incubation times.

Arabidopsis *mutants with altered adventitious root formation*

IBA is an important factor for adventitious root formation if applied exogenously. However, endogenous auxins may also play a role in the rooting process. Therefore three mutants with altered auxin levels were investigated for their ability to form adventitious roots after IBA treatment. The mutant *amt1* (Kreps and Town, 1992) has no altered phenotype compared with the wild type when grown under normal conditions. However, if *amt1* was grown on 10 μM IBA, the roots looked more stunted with a higher number of lateral roots and, at higher concentrations, less root growth than the wild type was observed. *amt1* also showed altered levels of IAA and IBA (Ludwig-Müller *et al*., 1993). It was therefore of interest to test whether this mutant behaved differently concerning adventitious rooting and so at the same time two other mutants with defects in the tryptophan biosynthesis pathway, *trp1* and *trp2* (Last *et al*., 1991; Rose *et al*., 1992) were included. Since adventitious root formation was shown to be concentration-dependent in *Arabidopsis*, several IBA concentrations were tested on wild-type and mutant stem segments. At intermediate IBA concentrations (3–10 μM), root induction was less efficient in *trp1*, a tryptophan auxotroph of *Arabidopsis* with a bushy phenotype but no demonstrable reduction in IAA levels, compared with wild-type Columbia (Fig. 8). The two other mutants (*amt1* and *trp2*) with measurably higher levels of IAA show root induction characteristics very similar to the wild type.

Discussion

Arabidopsis has been used for the investigation of lateral root development (Neuteboom *et al*., 1999) because of its relatively simple organization of both primary and lateral roots (Dolan *et al*., 1993). Lateral root formation in root cultures of *Arabidopsis* was initiated by exogenous auxin. Differential screening of a cDNA library from roots treated with 1-NAA and the inactive analogue 2-NAA led to the isolation of four cDNAs clones coding for proteins putatively active outside the cell such as subtilisin-like serine protease (Neuteboom *et al*., 1993). *Arabidopsis* mutants exhibiting more lateral roots (*sur1*, *sur2*) were linked to an overproduction of IAA (Boerjan *et al*., 1995; Delarue *et al*., 1998). However, other genes regulated independently of auxin induction are also involved in lateral root development, such as the nuclear-localized protein ALF4 (DiDonato *et al*., 2004).

Evidence for the involvement of IBA, but not IAA, in lateral root development was recently reported for lateral root induction in rice (Wang *et al*., 2003). While IBA was

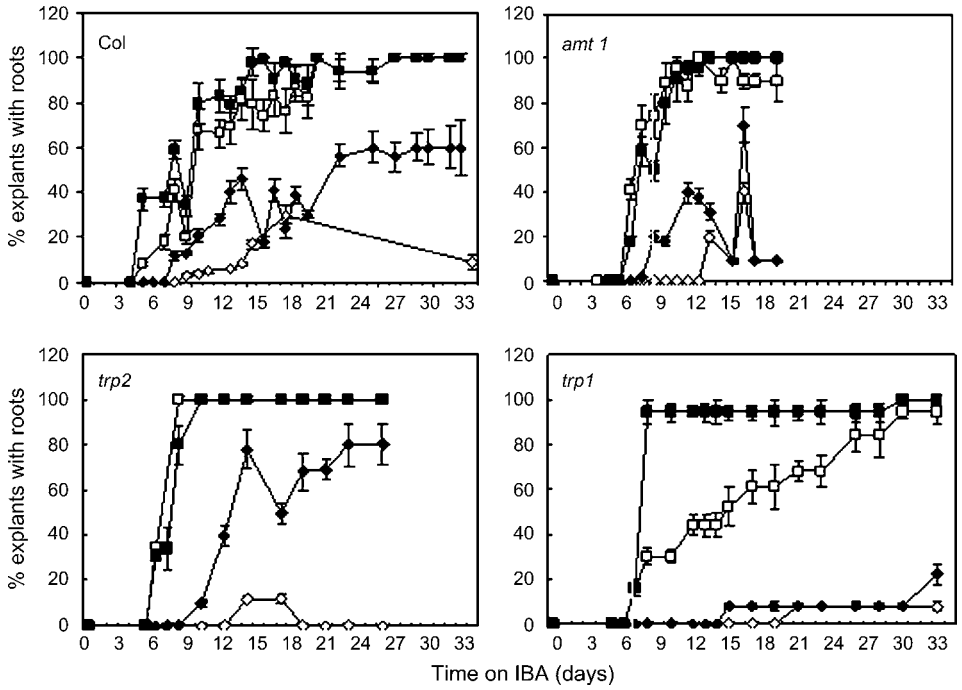

Fig. 8. Adventitious root induction at different IBA concentrations in wild type (Columbia) and three different *Arabidopsis* mutants with altered auxin content over a time period of 5 weeks: (open diamonds) 1 μM, (filled diamonds) 3 μM, (open squares) 10 μM, (filled squares) 30 μM.

able to induce lateral roots, the same response was found only at 20-fold higher concentrations of IAA (Chhun *et al*., 2003, 2004). In addition, a rice lateral rootless mutant *Lrt1* could be rescued by IBA but not IAA treatment (Chhun *et al*., 2003). The mutated gene has yet to be described.

In contrast to lateral root development, adventitious root formation has significant practical implications because of the many plant species that are difficult to root. IBA is now used commercially worldwide to root many plant species (Hartmann *et al*., 1990). However, *Arabidopsis* as a model to study adventitious rooting has so far been neglected. The aim of this study was 2-fold: (i) to analyse the process leading to adventitious roots on *Arabidopsis* stems and to find out which of the two auxins known to be present in *Arabidopsis* are involved in the process and to devise an experimental system which could be used to distinguish between callus and root formation and between IAA and IBA in the rooting process; (ii) to test this system for its use in the isolation of differentially expressed transcripts specifically involved in the rooting process. These transcripts could allow a more detailed analysis of adventitious rooting at the molecular level and help to identify candidate genes important for this process. The possible function of the transcripts isolated in this study for the rooting process will be briefly discussed. Furthermore, this system is also suitable for the analysis of available *Arabidopsis* mutants or chemical inducers or inhibitors of the rooting process.

It was shown that IAA and IBA were able to induce adventitious roots on cuttings of *Arabidopsis* stems if the segments were not removed during the treatment (Fig. 1), whereas removal of the segments from auxin-containing medium to MS medium only resulted in the production of calli with about the same efficiency for both hormones. Callus formation preceded adventitious rooting (Fig. 2). After shorter incubation times only IBA treatment resulted in the formation of roots (Fig. 3), indicating that IBA is an important factor for rooting. Several possibilities exist to explain the better performance of IBA versus IAA (summarized in Epstein and Ludwig-Müller, 1993): (i) higher stability, (ii) differences in metabolism, (iii) differences in transport, and (iv) IBA is a slow release source of IAA.

There is now a great deal of evidence that IBA occurs naturally in plants. The higher stability of IBA, in contrast to IAA, during rooting assays was reported by Nordström *et al*. (1991) which affected both degradation and metabolism. It was therefore suggested that IBA may be a very simple 'conjugate' of IAA and must be converted to IAA by β-oxidation to have an auxin effect. The conversion of IBA to IAA occurs in many plant species, such as *Malus pumila* (Alvarez *et al*., 1989), *Pinus sylvestris* (Dunberg *et al*., 1981), *Populus tremula* (Merckelbach *et al*., 1991), *Pyrus communis* (Baraldi *et al*., 1993), and *Vitis vinifera* and *Olea europaea* (Epstein and Lavee, 1984). However, in microcuttings of *Malus* it was found that IBA was converted to IAA only at very low levels (1%), but IBA itself induced more roots than IAA. This led the authors to suggest that either IBA itself is active or that it modulates the activity of IAA (van der Krieken *et al*., 1992, 1993).

The transport hypothesis is supported by recent findings that IBA and IAA are differently transported in *Arabidopsis* (Rashotte *et al*., 2003). These experiments are in agreement with this study's results using polar auxin transport inhibitors.

Several lines of evidence are now emerging which suggest that part of the effects of IBA are the direct action of the auxin itself (Ludwig-Müller, 2000; Poupart and Waddell, 2000), although other functions may be modulated by the conversion of IBA to IAA via β-oxidation (Zolman *et al*., 2000; Bartel *et al*., 2001). For example, drought and osmotic stress induced the synthesis of IBA and, consequently, the endogenous content of IBA was increased, whereas IAA was less affected (Ludwig-Müller *et al*., 1995). In addition, IBA but not IAA was induced after the inoculation of maize roots with an arbuscular mycorrhizal fungus (Ludwig-Müller *et al*., 1997; Kaldorf and Ludwig-Müller, 2000). In this paper a system was established for the induction of adventitious roots on sterile-grown stem sections of *Arabidopsis thaliana* where IBA induced adventitious roots under conditions where IAA was ineffective (Fig. 3). There was a desire to dissect the rooting process and therefore different time and concentration schemes were used for the optimization of adventitious root formation (Fig. 4), which allowed callus and subsequent root formation to be distinguished (Fig. 5).

The second goal of this research was the identification of differentially expressed transcripts during the rooting process. For this, the differential induction of callus and root on *Arabidopsis* stem segments were used and those treatments were compared with the controls (Fig. 6). Only those transcripts which showed up under treatment C (Fig. 6) were analysed further.

Initial studies on the hydrolytic enzymes found during root formation after IBA treatment in cuttings of mung bean revealed the induction of endo-β-1,4-glucanase (Shoseyov *et al*., 1989), whereas the activities of β-1,3-glucanase and α-amylase were not affected. It was shown by *in situ* hybridization that the genes for endo-β-1,4-glucanase were expressed in the area of adventitious root primordia formation and in the cortex, where maceration of the cell walls was in progress in order to enable root emergence through the hypocotyl. To detect the induction of genes during adventitious root formation in loblolly pine (*Pinus taeda*) after treatment with IBA, a non-targeted approach via differential display reverse transcription-polymerase chain reaction was carried out (Hutchison *et al*., 1999). One of the clones isolated by this method showed strong similarity to the α-expansin gene family of angiosperms and the differential gene expression after IBA treatment was confirmed by RNA blot analysis. Expansins are thought to be responsible for acid-induced cell wall loosening and are expressed in rapidly growing tissues (Cosgrove and Li, 1993; McQueen-Mason, 1995). They were reported to be induced in loblolly pine in non-growing regions of the stem prior to the resumption of cell division leading to the appearance of adventitious roots (Hutchison *et al*., 1999).

One fragment differentially expressed during the adventitious rooting process in *Arabidopsis* (Fig. 6B) was identified as a regulatory subunit B of protein phosphatase 2A. In plants, type 2A serine/threonine protein phosphatases (PP2As) are critical in controlling the phosphorylation state of proteins involved in such diverse processes as metabolism, cell–cell communication, response to hormone, and auxin transport (Smith and Walker, 1996). The specificity, activity and subcellular targeting of PP2A is modulated by its association with the A and B subunits (Kamibayashi *et al*., 1994). In *Arabidopsis*, three families of B-type regulatory subunits were identified, each consisting of more than one member (Corum *et al*., 1996; LaTorre *et al*., 1997; Rundle *et al*., 1995; Sato *et al*., 1997). Expression analysis indicated that, in plants, every B subunit shows a widespread, but fine-tuned, expression pattern in different organs (Thakore *et al*., 1999). The function of PP2A during polar auxin transport has recently received more attention (Muday and DeLong, 2001, and references therein). One *Arabidopsis* mutant that provided insight into the regulation of auxin transport is called *roots curl in NPA1* (*rcn1*). This mutant was isolated using an assay for alterations in differential root elongation in the presence of the auxin transport inhibitor NPA aimed at isolating genes encoding proteins involved in auxin transport or its regulation. The *RCN1* gene encodes a regulatory A subunit of PP2A and the *rcn1* mutant exhibits reduced PP2A activity in extracts (Deruère *et al*., 1999). The phenotypic alterations in this mutant are consistent with reductions in PP2A activity because treatment of wild-type plants with the phosphatase inhibitor cantharidin produces a phenocopy of *rcn1*. The *RCN1* gene is expressed in the seedling root tip, the site of basipetal transport, in lateral root primordia, and in the pericycle and stele, the likely site of acropetal transport (Muday and DeLong, 2001). It can be hypothesized that other PP2A subunits are co-ordinately expressed and that polar auxin transport also plays a role in adventitious root formation in *Arabidopsis*. This assumption is supported by the observation here that the auxin transport inhibitor TIBA inhibited adventitious root formation. Deduced from the findings summarized above a role can be proposed for PP2A in the regulation of auxin transport during adventitious rooting by altering the phosphorylation status of proteins involved in these processes thus most likely acting upstream of auxin transport. Auxin transport itself might be important for adventitious rooting by increasing local auxin concentrations.

A second fragment was identified as derived from an early-responsive dehydration stress ERD3 with otherwise unknown function (http://www.tigr.org/tdb/e2k1/ath1/). The sequence contains also a methyltransferase motif. Protection against dehydration may result in an increase

of lateral or adventitious root formation. It was shown that IBA synthesis was increased under drought stress in maize (Ludwig-Müller *et al*., 1995) and the root system under these conditions was shorter, but with considerably more lateral roots. Drought rhizogenesis is an adaptive strategy that occurs during progressive drought stress and is characterized in *Arabidopsis* and other Brassicaceae and related families by the formation of short tuberized hairless roots (Vartanian *et al*., 1994). These roots are capable of withstanding a prolonged drought period and give rise to a new functional root system upon rehydration. IBA might play a role during this process by inducing new roots. This protein might therefore play a more general role in IBA-induced root formation. As long as the function of ERD3 is unclear, this has to remain a hypothesis.

The Histidine Triad (HIT) motif identified in the third gene product, His-phi-His-phi-His-phi-phi (phi, a hydrophobic amino acid), was identified as being highly conserved in a variety of organisms (Seraphin, 1992). The crystal structure of rabbit Hint (histidine triad nucleotide-binding protein), purified as an adenosine and AMP-binding protein, showed that proteins in the HIT superfamily are conserved as nucleotide-binding proteins (Brenner *et al*., 1997). Hint homologues hydrolyse adenosine 5′ monophosphoramide substrates and function as positive regulators of Cdk7/Kin28 *in vivo* (Bieganowski *et al*., 2002), and Fhit (fragile histidine family) homologues related to the HIT family are diadenosine polyphosphate hydrolases (Barnes *et al*., 1996). Therefore, the role of this protein during adventitious root formation might be in the regulation of the cell cycle or in signal transduction pathways.

In conclusion, it has been shown that it was possible to dissect the adventitious root formation process in *Arabidopsis* in such a way as to distinguish between the action of the two auxins IAA and IBA and to establish conditions where one hormone treatment arrests the process at the callus formation stage, whereas a second hormone treatment induces the formation of roots from these calli. In addition, it has been shown that the experiments presented here are a promising method to identify IBA-induced transcripts during adventitious root formation in the model plant *Arabidopsis thaliana*. To study the process of adventitious root formation further, several experiments can be envisioned: (i) the isolation of additional differentially expressed fragments from this screen, or using the now available microarrays to increase the number of cDNAs; (ii) using this screening method to identify *Arabidopsis* mutants impaired in adventitious root formation; and (iii) using known *Arabidopsis* mutants to investigate their response to IBA in this system. The gene sequences identified can then be used to probe the adventitious rooting pathway in horticulturally important species that are difficult to root.

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Indole-3-butyric acid synthesis in ecotypes and mutants of *Arabidopsis thaliana* under different growth conditions

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Summary

Although IBA is a naturally occurring auxin, its role in plant development is still under debate. In this study a set of *Arabidopsis* mutants was used to analyze the biosynthesis of IBA in vitro. The mutants chosen for this study can be classified as: (1) involvement in auxin metabolism, transport or synthesis (*amt1*, *aux1*, *ilr1*, *nit1*, *rib1*, *sur1*, *trp1-100*); (2) other hormones possibly involved in the regulation of IBA synthesis (*aba1*, *aba3*, *eto2*, *fae1*, *hls1*, *jar1*); (3) photomorphogenesis (*det1*, *det2*, *det3*); and (4) root architecture (*cob1*, *cob2*, *scr1*). In addition, two transgenic lines overexpressing the IAA glucose synthase (*iaglu*) gene from maize were analyzed. The ecotypes No-0 and Wassilewskija showed the highest IBA synthetase activity under control conditions, followed by Columbia, Enkheim and Landsberg erecta. In the mutant lines IBA synthetase activity differed in most cases from the wild type, however no particular pattern of up- or down-regulation, which could be correlated to their possible function, was found. For *rib1* mutant seedlings it was tested whether reduced IBA synthetase activity correlates with the endogenous IBA levels. Free IBA differed only depending on the culture conditions, but gave no clear correlation with IBA synthetase activity compared to the wild type. Since drought and osmotic stress as well as abscisic acid (ABA) application enhanced IBA synthesis in maize, it was tested whether IBA synthetase from *Arabidopsis* is also inducible by drought stress conditions. This was confirmed for the two ecotypes Col and Ler which showed different IBA synthetase activity when cultivated with various degrees of drought stress. IBA synthetase was also determined in photomorphogenic mutants under different light regimes. Induction of IBA synthetase in *det1* and *det3* plants

Abbreviations: ABA, abscisic acid; acetyl-CoA, acetyl coenzyme A.; 2; 4-D, 2, 4-dichlorophenoxyacetic acid; GC-MS, gas chromatography-mass spectrometry; HPLC, high performance liquid chromatography; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; JA, jasmonic acid; NAA, 1-naphthylacetic acid

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was found under short day plus a red light pulse or in the dark, respectively. The results are discussed with respect to the functions of the mutated genes.

Introduction

Auxins are a class of phytohormones which are involved in many aspects of growth and development of plants (Davies, 1995). In recent years it has becoming more clear that besides the 'classical' auxin indole-3-acetic acid (IAA) other auxin-like substances exist in plants such as indole-3-butyric acid (IBA) (Ludwig-Müller, 1999, 2000). Some of them are even more effective as an auxin than IAA during certain developmental stages or in certain plant species (Epstein and Ludwig-Müller, 1993), whereas others show only weak auxin activity (Ludwig-Müller, 1999; Ludwig-Müller and Cohen, 2002).

IBA has been identified by modern techniques such as gas chromatography-mass spectrometry in a wide variety of plants (Ludwig-Müller, 2000). Levels of IBA vary with different growth conditions such as pH of the growth medium, light intensity and volume of the culture flask as shown for aseptically grown *Arabidopsis thaliana* seedlings (Ludwig-Müller et al., 1993). While IAA occurred in *Arabidopsis* mostly as amide conjugates, higher amounts of IBA ester conjugates were found in *Arabidopsis* (Ludwig-Müller et al., 1993). However, labeled IBA was effectively metabolized to both ester and amide conjugates (Ludwig-Müller and Epstein, 1993). A UDP-glucose transferase from *Arabidopsis* was shown to be capable to synthesize IAA- and IBA-glucose (Jackson et al., 2002).

Zea mays seedlings were found to convert IAA to IBA in vivo (Ludwig-Müller and Epstein, 1991). The in vitro conversion of IAA to IBA in maize seedlings is dependent on a microsomal membrane preparation which forms IBA in the presence of acetyl-CoA and ATP (Ludwig-Müller et al., 1995a). This reaction was also found in a wide variety of other plant species (Ludwig-Müller et al., 1995a). The enzyme termed IBA synthetase was characterized in maize and partially purified (Ludwig-Müller and Hilgenberg, 1995). The rate of IBA synthesis in maize seedlings was dependent on the culture conditions of the plants. When seedlings were exposed to drought stress, IBA synthetase activity increased dramatically, whereas high amounts of water (= hypoxic stress) inhibited IBA synthesis (Ludwig-Müller et al., 1995b). The endogenous free IBA content showed the same pattern as the IBA synthetase activity. Endogenous ABA also increased in plants cultivated under drought stress. Since the application of high ABA concentrations also promoted IBA synthetase activity, it was concluded that drought stress may act on IBA synthesis via the increase of endogenous ABA.

Since it was shown that IBA can also be converted to IAA (Epstein and Lavee, 1984), the question arose whether IBA acts as auxin itself or through its conversion to IAA. For both possibilities experimental evidence has been reported. Evidence for independent auxin activity of IBA comes from lateral and adventitious rooting experiments. It was shown that IBA induced adventitious roots on sterile grown stem sections of *A. thaliana* at concentrations where IAA was uneffective (Ludwig-Müller et al., 2005a). IBA was also more effective to promote lateral roots at lower concentrations compared to IAA in *Arabidopsis* (Zolman et al., 2000). If IBA needs to be converted to IAA, it should be active at higher concentrations than IAA. In a lateral rootless rice mutant (*Lrt1*; Chhun et al., 2003) exogenous application of IBA but not IAA restored both auxin-specific phenotypes, namely lateral roots and gravitropic response, while IAA rescued only the gravitropic response, again indicating that the function of these two auxins can be separated. In addition, a variety of stresses (drought and osmotic stress, herbicide treatment) induced the synthesis of IBA and consequently the endogenous content of IBA was also increased, whereas IAA was less affected (Ludwig-Müller et al., 1995b,2000). Also, after inoculation of maize roots with an arbuscular mycorrhizal fungus, the levels of IBA, but not IAA, were elevated (Ludwig-Müller et al., 1997). This coincided with a root phenotype that could be mimicked by exogenously applied IBA (Kaldorf and Ludwig-Müller, 2000). Finally, a mutant screen for IBA resistant mutants produced several classes with differential resistance to IAA and IBA (Poupart and Waddell, 2000; Zolman et al., 2000).

Several IBA-resistant mutants (*ibr*) were recovered in a root elongation screen using *Arabidopsis*. Some mutants showed IAA-resistant root elongation and corresponded to auxin response mutants, the others had IAA-sensitive root elongation and were therefore classified as IBA response mutants (Zolman et al., 2000). From these mutants four different classes were identified: class I consisted of mutants with defects in ß-oxidation of fatty acids and IBA, class II and III of mutants with

defects in ß-oxidation of IBA only resulting in different root phenotypes, and class IV mutants in IBA perception, transport or response (Zolman et al., 2000). The differences in IAA and IBA responses may be indicators that IBA acts, at least in part, through its conversion to IAA via ß-oxidation.

The *rib1* (resistant to IBA) mutant of *Arabidopsis* was recovered in a screen for root gravitropic mutants and discriminates between IAA and IBA (Poupart and Waddell, 2000). The response of mutant seedlings to different auxins was examined in a root elongation assay. *rib1* was resistant to IBA and 2,4-D, but had a wild-type response to IAA and NAA. In addition, it is slightly hypersensitive to ABA which might point to a cross-talk between IBA and ABA. It was concluded that the *rib1* mutation specifically affects IBA response in mutant plants. *rib1* mutants also showed an increase in hypocotyl length and number of lateral roots but mature plants were phenotypically indistinguishable from wild-type. The finding that resistance to IAA and IBA in plants is genetically separable supports the theory that IBA plays a specific role in root development and response to environmental stimuli.

Despite these recent findings, the role of IBA in vivo is still not clear. The elucidation of how IBA is made within the plant is one step towards understanding how it may function in plant development. Although the enzyme(s) involved in the conversion of IAA to IBA have been characterized and partially purified from maize, it was not possible to obtain a pure protein fraction. IBA synthetase from light-grown seedlings was shown to be a small protein (MW ca. 30 kDa) with an acidic isoelectric point, and is most likely located on the membranes of the endoplasmic reticulum (Ludwig-Müller and Hilgenberg, 1995; Ludwig-Müller et al., 1995a).

Since the biosynthesis of IBA from IAA has been confirmed also in seedlings and mature plants of *A. thaliana* (Ludwig-Müller and Epstein, 1994; Ludwig-Müller and Hilgenberg, 1995), a small set of *Arabidopsis* ecotypes and mutants was used to analyze variations in IBA synthetase activity. Mutants with altered enzyme activity may be valuable tools to help isolating the enzyme(s) and/or genes involved and to study the regulation of IBA synthesis.

Materials and methods

Plant material

All *Arabidopsis* material used in this study is summarized in Table 1. *Arabidopsis* seeds were surface sterilized with 30% commercial bleach (Clorox), washed with excess sterile water and then used in the various culture conditions. For comparable IBA synthetase measurement *Arabidopsis* plants were grown on filter paper soaked with sterile tap water. To improve the harvest of roots, the filter paper was covered in most of the experiments with a nylon membrane (20 µm pore size), where the roots could not grow through. The plants were cultivated in trays of 17 × 27 cm (459 cm^2). Ca. 2000 seeds per tray were distributed on four layers of filter paper which were soaked with different amounts of sterile water (200, 150, 90, 50, and 30 mL) as previously described (Ludwig-Müller et al., 1995b). Plants were grown under continuous illumination. For some experiments long day (16:8 h light:dark cycle), short day (8:16 h light:dark cycle) and short day with an intermediate 1 h red light pulse (maximum wavelength 660 nm) in the middle of the dark period as well as dark grown plants were used. The seedlings were routinely harvested after 7 d for enzyme measurements.

For IBA determination the seedlings were grown in addition to the filter paper conditions on agar plates containing either MS (Murashige and Skoog, 1962) or Gamborg (Gamborg et al., 1968) medium or in liquid culture using MS medium (Ludwig-Müller et al., 1993). Seedlings were harvested after 10 or 5 d, respectively.

Mature plants were sown on a compost:peat:sand (3:1:1) mixture, kept for 48 h at 4 °C and then grown under a 16 h light:8 h dark cycle at 23 °C under 70% humidity. After four weeks of culture, the watering treatments were started as given in Results. Treatment was continued for two additional weeks.

Enzyme preparation

The plant material was harvested and homogenized with a mortar and a pestle using 100 mM HEPES buffer, pH 7, containing 1 mM $MgSO_4$. The homogenate was filtered and centrifuged at 10 000*g* for 10 min to remove cell particles. The supernatant was then centrifuged at 50 000*g* for 60 min and resuspended in HEPES buffer (1 mL 10 g^{-1} fresh weight). All operations were carried out at 4 °C (Ludwig-Müller and Hilgenberg, 1995; Ludwig-Müller et al., 1995a).

Enzyme assay for IBA formation

The enzyme assay has been carried out according to Ludwig-Müller et al. (1995a). Briefly, the

Table 1. Plant material used in this study

Line	Wild type	Phenotype description	Reference
Columbia		Ecotype	
amt1	Col	α-methyltryptophan resistant; anthranilate synthase α-subunit	Kreps et al. (1996)
cob1	Col	Altered root architecture	Benfey et al. (1993)
cob2	Col	Altered root architecture	Benfey et al. (1993)
det1	Col	De-etiolated	Chory and Peto (1990)
det2	Col	De-etiolated; steroid-5α-reductase	Chory et al. (1991)
det3	Col	De-etiolated; vacuolar ATP synthase subunit	Cabrera et al. (1993), Schumacher et al. (1999)
eto2	Col	Ethylene overproducing; ACC synthase	Vogel et al. (1998)
fae1	Col	Fatty acid elongation, condensing enzyme	Lemieux et al. (1990)
hls1	Col	Hookless (reduced ethylene production, N-acetyltransferase deficient)	Guzman and Ecker (1990), Lehman et al. (1996)
jar1	Col	Jasmonic acid insensitive; conjugation of JA	Staswick et al. (1992, 2004)
nit1	Col	IAA biosynthesis; nitrilase1	Normanly et al. (1997)
sur1 (allelic to *rty1*, *hls3*, *alf1*)	Col	Root proliferation (increased amount of IAA)	Delarue et al. (1999)
trp1-100	Col	Tryptophan biosynthesis; phosphoribosyl-anthranilate transferase	Rose et al. (1997)
WD8	Col	35SZmIAGLU	Ludwig-Müller et al. (2005b)
WD15	Col	35SZmIAGLU	Ludwig-Müller et al. (2005b)
Landsberg erecta	La	Erecta mutation	
aux1	Ler	Putative auxin influx carrier	Bennett et al. (1996), Pickett et al. (1990)
aba1	Ler	ABA deficient; epoxidation of zeaxanthin	Rock and Zeevaart (1991a)
aba3	Ler	ABA deficient; oxidation of abscisic aldehyde to ABA	Schwartz et al. (1997)
Enkheim		Ecotype	
No-0		Ecotype	
rib1	No-0	IBA resistant	Poupart and Waddell (2000)
WS		Ecotype	
ilr1	WS	IAA-leucine resistant; IAA amidohydrolase	Bartel and Fink (1995), LeClere et al. (2002)
scr1	WS	Altered root architecture	Wysocka-Diller et al. (2000)

All ecotypes and mutants if not stated otherwise were either obtained from the Arabidopsis Biological Resource Center (ABRC), Ohio, USA or the Nottingham Arabidopsis Seed Stock Center (NASC), England. The mutant *rib1* was obtained from Candace Waddell, McGill University, Montreal, Canada.

reaction mixture contained, in a total volume of 500 µL, 100 µL microsomal membranes, 2 mM acetyl-CoA, and 6 mM ATP. The reaction was started by the addition of non-labeled IAA at a final concentration of 1 mM. The reaction was carried out at pH 7 and incubation time was routinely 1 h at room temperature. Linearity of the enzyme reaction was observed for up to 4 h in maize (Ludwig-Müller and Hilgenberg, 1995) and *Arabidopsis* (ecotypes Columbia and Landsberg; J. Ludwig-Müller, unpublished results). The reaction was stopped by adding 30 µL 1 N HCl, followed by subsequent extraction of the aqueous phase with 500 µL of ethyl acetate. The organic phase was removed, evaporated to dryness, and resuspended in 20 µL methanol. The sample was kept in liquid nitrogen for HPLC analysis. The enzymatic activity is either expressed as µg IBA formed per ml and min or µg IBA per mg protein and min. Protein determination was carried out using the BCA protein assay reaction kit (Pierce Chemicals, Rockford, IL, USA) with bovine serum albumin as standard.

HPLC analysis

The total methanol extract (20 µL) was subjected to HPLC (Biotronik BT 8100, Maintal, Germany), equipped with a 4.6 × 125 mm Lichrosorb C_{18}, 5 µ, reverse phase column. As solvent, 52% methanol containing 1% aquous acetic acid was used at a flow rate of 0.7 mL min^{-1}. Detection of IBA was performed at 280 nm by co-chromatography with an authentic standard. The amount of IBA

(R_t 12.2 min) enzymatically formed was determined using a standard curve and all values were corrected on an internal standard with known concentrations of IAA (R_t 7.9 min).

Determination of endogenous IAA and IBA

The plant material was harvested, extracted and purified as described in Chen et al. (1988) using NH_2-columns (J.T. Baker). One hundred ng $^{13}C_1$-IBA (Sutter and Cohen, 1992) and 200 ng $^{13}C_6$-IAA (Cambridge Isotope Laboratories) were added as internal standard to each sample. After elution from the NH_2-columns the samples were concentrated and subjected to HPLC-purification. The same system as for IBA identification in the enzyme assay was used. The IAA- and IBA-corresponding peaks were collected, evaporated to dryness, taken up in 100 µL ethyl acetate and methylated with diazomethane (Cohen, 1984) for GC-MS analysis.

Analysis was performed using a Varian Saturn 2100 Ion Trap MS system using electron impact ionisation at 70 eV, coupled to a Varian CP-3900 GC equipped with an Varian CP-8400 autosampler (Varian, Walnut Creek, CA, USA). For the analysis 2.5 µL of the methylated sample dissolved in 20 µL ethyl acetate was injected in the splitless mode (splitter opening 1:100 after 1 min) onto a Phenomenex ZB-5 column, 30 m × 0.25 mm × 0.25 µm (Phenomenex, Aschaffenburg, Germany) using He carrier gas at 1 mL min^{-1}. Injector temperature was 250 °C and the temperature program was 70° for 1 min, followed by an increase of 20 min^{-1} to 280 °C, then 5 min isothermically at 280 °C. Transfer line temperature was 280 °C. Scan rate was 0.6 s scan^{-1}, multiplier offset voltage 200 V, emission current 30 µA and the trap temperature 200 °C. For higher sensitivity for the quantification of endogenous auxins the µSIS mode (Varian Manual) was used monitoring the molecular and quinolinium ions at m/z 189/195 and 130/136 for IAA and m/z 217/218 and 130/131 for IBA, respectively (ions deriving from endogenous $^{13}C_6$-IAA and $^{13}C_1$-IBA). The methyl esters of IAA and IBA eluted at 10.4 and 11.2 min, respectively, under these conditions. The endogenous levels of free auxins were calculated by the isotope dilution equation as given by Ilić et al. (1996).

Results

Various *Arabidopsis* ecotypes and mutants show differences in IBA synthetase activity

A small collection of *Arabidopsis* ecotypes and mutants (Table 1) was screened for differences in their capability to synthesize IBA from IAA as precursor. The mutants were selected for (a) phenotypes associated with altered auxin levels, (b) phenotypes associated with other plant hormones that may interact with auxin biosynthesis, (c) phenotypes associated with photomorphogenesis and (d) phenotypes with altered root architecture (for the different phenotypes see also Table 1 and discussion).

Since there is considerable influence of environmental factors on IBA synthetase activity (Ludwig-Müller et al., 1993; Ludwig-Müller et al., 1995b), the growth conditions for the set of mutants investigated (except the *det* mutants during different light regimes and the variations during water stress) were kept under tightly controlled conditions (same stage of seedling development [cotyledon stage]; controlled temperature and controlled light conditions as well as controlled amount of water). During seedling growth the IBA synthetase activity increased up to day five in *Arabidopsis* under the chosen conditions, then up to day 10 there was no change in activity and after that the activity decreased under the chosen conditions (data not shown). So the experiment with mutants was done during the period of lowest variation of IBA synthetase activity in the seedlings at the cotyledon stage. Col plants cultivated in soil showed no significant variations in IBA synthetase activity between two and 5 weeks after sowing, only after 6 weeks the enzyme activity increased by about 3-fold (data not shown). This also indicates constant conditions during the experiments with plants grown in soil (see Fig. 3).

The ecotypes with highest IBA activity, expressed as µg IBA mg protein^{-1}min^{-1}, were No-0 (0.35 ± 0.09) and Wassilewskija (WS) (0.33 ± 0.07) followed by Columbia (Col) (0.15 ± 0.03), Enkheim (En) (0.07 ± 0.01) and Landsberg erecta (Ler) (0.02 ± 0.001), respectively. From the mutants with Col background, *cob1*, *det1*, *det2*, *det3*, *hls1* and the IAA-glucose synthase overexpressing transgenic line WD15 showed significantly increased IBA synthetase activity compared to the wild type (Table 2). No differences were found for *amt1*, *fae1*, *nit1*, *jar1* and the IAA-glucose synthase overexpressing transgenic line WD8 (data not shown), whereas in *cob2*, *eto2*, *sur1* and *trp1-100* seedlings the activity was lower than in the wild type (Table 2). Compared to their Ler background, an increased IBA synthetase activity was found in *aux1* (4.5-fold), *aba1* (2-fold) and *aba3* (18-fold). The mutant *scr1* showed no different IBA synthetase activity compared to the wild type WS and the IBA synthetase activity in *ilr1* was only slightly

Table 2. IBA synthetase activity in various ecotypes and mutants of *Arabidopsis thaliana* under controlled growth conditions (90 mL H_2O per tray, 16 h light:8 h dark cycle)

Phenotype	Mutant	Ecotype	IBA synthetase activity (μg IBA mg protein^{-1}min^{-1})	Percent of respective wild type
Ecotypes	Columbia		0.15±0.03	100
	Landsberg erecta		0.02±0.001	100
	No-0		0.35±0.09	100
Altered auxin levels, transport, metabolism	*aux1*	Ler	0.09±0.025	450
	rib1	No-0	0.1±0.03	29
	rty1/sur1	Col	0.04±0.006	27
	trp1-100	Col	0.006±0.001	4
	WD15	Col	0.91±0.27	607
Other plant hormones	*aba1*	Ler	0.04±0.007	200
	aba3	Ler	0.51±0.09	2550
	eto2	Col	0.02±0.005	13
	hls1	Col	0.31±0.08	207
Photomorphogenesis	*det1*	Col	0.5±0.13	333
	det2	Col	0.31±0.07	207
	det3	Col	0.68±0.15	453
Altered root architecture	*cob1*	Col	0.78±0.24	520
	cob2	Col	0.003±0.00004	2

For the different phenotypes see also Table 1 and discussion. Seven day old seedlings were sown on filter paper covered by a nylon membrane and harvested at the cotyledon stage. Values are given as means±SE of three independent experiments.

higher (data not shown). Compared to No-0 the IBA synthetase activity in the IBA-resistant mutant *rib1* was reduced by about 70% (Table 2).

Is there a correlation between reduced or increased IBA synthetase activity and endogenous IBA levels? As an example the *rib1* mutant was chosen because this mutants is directly related to IBA. *rib1* had reduced IBA synthetase activity. The auxin levels in *rib1* seedlings were determined under different growth conditions. Free IAA levels varied between 108 and 156 ng g^{-1} fresh weight in the wild type No-0, depending on the growth conditions. Only in liquid culture, which does not correspond to the conditions under which IBA synthetase was measured, higher IAA levels (463 ng g^{-1} fresh weight) were found in the mutant *rib1*. On solid medium IAA levels seemed slightly lower in the mutant, but this was not significant. IBA levels were generally lower than IAA levels in both wild type and mutant seedlings. A slight increase in IBA levels when the seedlings were grown in liquid culture was also observed in *rib1* (129 ng g^{-1} frsh weight) in comparison to the wild type (81 ng g^{-1} fresh weight). On solid medium no consistent differences were observed. Therefore, it can be concluded that IAA and IBA levels are not directly correlated to the enzymatic activity of IBA synthetase.

The effect of light on IBA synthetase activity

Light seems to have an effect on IBA synthetase in maize seedlings (Ludwig-Müller et al., 1995a). Incubation of microsomal membranes from shoots and roots of dark-grown maize with labeled IAA resulted in the formation of an unknown labeled reaction product, whereas only low amounts of IBA was detected under these conditions. On the contrary, microsomal membranes from light-grown shoots and roots converted IAA directly to IBA.

The *det* mutants show a de-etiolated phenotype in the darkness and were therefore used to monitor IBA synthetase activity under different light regimes (Fig. 1). All *det* mutant seedlings showed an increased IBA synthetase activity compared to the wild type when grown under continuous light (Table 2). Other light regimes used included typical long day conditions (16 h light: 8 h dark cycle), short day conditions (8 h light:16 h dark cycle), the third treatment had a photomorphogenically active red light pulse of 1 h during the dark period and finally the plants were grown completely in the dark (Fig. 1). In the wild type Col no significant differences in IBA synthetase activity were induced by the different treatments, although the *det1* and *det3* mutant plants showed some differences in IBA synthetase activity. While *det1* plants showed an

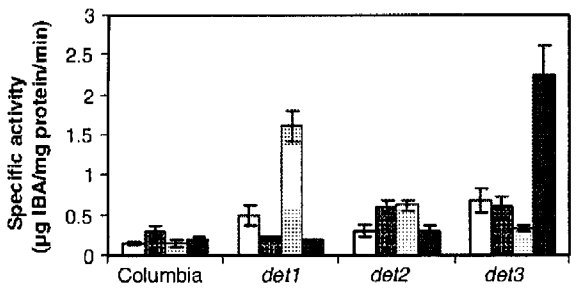

Figure 1. IBA synthetase activity in photomorphogenetic *det* mutants compared to wild type. The conditions were long day (16 h light:8 h dark cycle) (□), short day (8 h light:16 h dark cycle) (▨), short day with a 1 h red light pulse (maximum wavelength 660 nm) in the middle of the dark period (▩), and complete darkness (■). Values are given as means ± SE of three independent experiments.

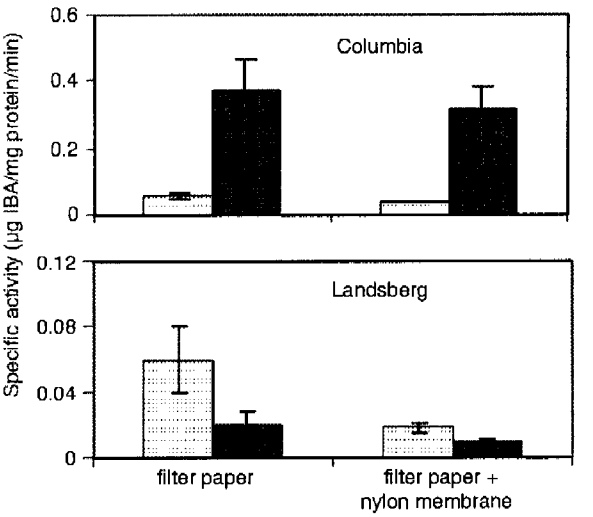

Figure 2. Comparison of IBA synthetase activity from Columbia and Landsberg erecta seedlings under two different watering conditions. Seven day old seedlings were sown on filter paper which were covered in one of the experiments with a nylon membrane and grown under continuous light. (▩) 50 mL H_2O; (■) 90 mL H_2O. Values are given as means ± SE of three independent experiments.

increase in IBA synthetase activity under short day plus red light treatment by about 3-fold compared to the other growth conditions, in *det3* plants darkness induced an increase in IBA synthetase activity by about 3-fold. Interestingly, the short day plus red light pulse treatment decreased the activity in *det3* plants compared to long day growth conditions. In *det2* plants both short day and short day plus red light slightly induced IBA synthetase activity.

The effect of water stress on IBA synthetase activity

When maize seedlings were grown on filter paper with different amounts of water, they exhibited differences in the activity of IBA synthetase with higher activities under more dry conditions. High amounts of water, which create a hypoxic environment, on the contrary inhibited IBA synthesis (Ludwig-Müller et al., 1995b). Since it was shown that this effect was not only observed in maize but also in wheat, pea and amaranth (Ludwig-Müller et al., 1995b), it was investigated whether this regulation could also be observed in *Arabidopsis*. Since it was difficult to harvest 7-day-old *Arabidopsis* seedlings from filter paper (most of the roots could not be recovered), an experiment was conducted where the filter paper was covered by a nylon membrane with 20 µm mesh, where the roots of the *Arabidopsis* plants did not grow through making it very easy to harvest the plant material. Two different ecotypes (Col and Ler) were tested for their IBA synthetase activity under the two conditions using 50 and 90 mL H_2O per tray (Fig. 2). The former condition was defined as drought condition which could induce IBA synthetase activity in maize, whereas the latter was used as well watered control condition (Ludwig-Müller et al., 1995b). It was not important whether the filter paper was used alone or covered with the membrane, because the effects were clearly visible under both conditions. The two ecotypes behaved completely different under the two watering conditions (Fig. 2). While Ler showed an increase in IBA synthetase activity under drought conditions, Col had higher IBA synthetase activity under control conditions.

To establish a different system for investigating the effect of drought on IBA synthetase activity in *Arabidopsis*, pot cultures with older plants grown in soil were used. Drought stress was created by two different treatments: (a) constant amount of water (30 mL per pot) at different time intervals (Fig. 3A) and (b) constant watering intervals (two times a week) with different amounts of H_2O (Fig. 3B). The pot weight was measured to obtain a value for the relative water content (black circles in Fig. 3). In treatment (a) the plants were watered either once a week, twice a week, three times a week, or daily with a constant amount of water. This treatment resulted in a linear decrease in water content in the pot from daily watering to watering only once a week (Fig. 3A). IBA synthetase activity showed high variability in individual samples under this treatment, therefore no significant differences were found in Col and Ler. However, there was a slight

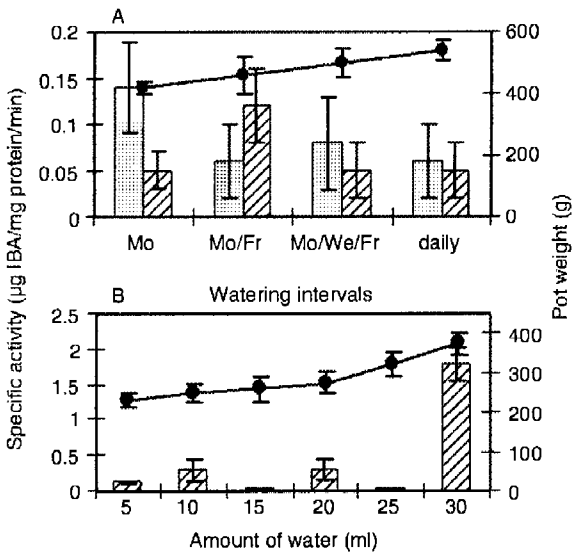

Figure 3. The effect of different watering regimes on IBA synthetase activity. The pot weight was used as a measure for soil moisture. A. A constant amount of water (30 mL) was given at different time intervals. B. Constant watering intervals (two times a week) were used with different amounts of H_2O. The second experiment was only done with Landsberg erecta. (○) Col; (□) Ler; (●) Pot weight. Values are given as means±SE of three independent experiments.

trend for Col plants having a higher IBA synthetase activity when the plants were only watered once a week, which was contrary to the findings obtained on filter paper/nylon membrane. Ler plants showed a slight increase in IBA synthetase activity when the plants were watered twice a week which would probably correspond to the 50 mL H_2O per tray treatment on filter paper. In the second experimental setup the plants were watered twice a week with different amounts of water to further increase drough conditions (Fig. 3B). The individual activity values were much more consistent under these conditions, so the activities became more comparable. It became clear that IBA synthetase activity was not further increased in Ler plants when the drought stress situation increased. On the contrary, highest activity was found at 30 mL of water whereas with less watering the activity also decreased.

Discussion

The biosynthesis of IBA in vitro has been demonstrated in several different plants and has been investigated in detail in maize (Ludwig-Müller and Hilgenberg, 1995; Ludwig-Müller et al., 1995a, b). The question of its role in vivo has been asked many times, but no sufficient explanation has been provided so far. IBA was found to be superior to IAA in the induction of adventitious roots on cuttings, but this has been attributed to the higher stability of IBA versus IAA (Nordström et al., 1991; Epstein and Ludwig-Müller, 1993). Recent data support the hypothesis that IBA can act partially through its interconversion to IAA but several other observed phenotypes may be due to IBA as an auxin itself (Ludwig-Müller, 2000; Zolman et al., 2000; Bartel et al., 2001; Ludwig-Müller et al., 2005a).

No *Arabidopsis* mutant has been dicovered so far that could be linked directly to IBA synthesis. The IBA-resistant mutants (*ibr*) isolated by Zolman et al. (2000) are either peroxisomal mutants, mutants probably defective in ß-oxidation of fatty acids and IBA and mutants defective in IBA signalling. The *rib1* mutant discovered in a similar screen (Poupart and Waddell, 2000) has characteristics which point to defects probably in the transport system. In addition, it was shown that IAA and IBA are transported differently (Rashotte et al., 2003).

From wheat (*Triticum aestivum*) an auxin amino acid conjugate hydrolase was identified which showed high specificity for IBA conjugates as substrates (Campanella et al., 2004). The hydrolysis of inactive auxin amide conjugates also contributes to the free IAA or IBA content. In *Arabidopsis*, although it was shown that IBA is conjugated to a high extent (Ludwig-Müller et al., 1993), no such activity for the conversion of IBA conjugates has been described so far. However, an UDP-glucose transferase was characterized from *Arabidopsis* which converts IAA and IBA to the respective glucose conjugates (Jackson et al., 2002).

The aim of this study was two-fold: (a) to investigate a set of *Arabidopsis* ecotypes and mutants for their capacity to synthesize IBA, and (b) to investigate the response of several mutants and ecotypes to environmental signals such as light conditions and drought stress. The mutant defects could be classified as follows: (1) involvement in auxin metabolism, transport or synthesis (*amt1, aux1, ilr1, nit1, rib1, sur1, trp1-100*); (2) other hormones possibly involved in the regulation of IBA synthesis (*aba1, aba3, eto2, fae1, hls1, jar1*); (3) photomorphogenesis (*det1, det2, det3*); (4) root architecture (*cob1, cob2, scr1*). In addition, two transgenic lines overexpressing IAA glucose synthase from maize were used (WD8, WD15).

Auxin synthesis, metabolism and transport

Two of the chosen mutants were previously shown to have higher levels of IAA (*rty1/sur1*;

Boerjan et al., 1995; Delarue et al., 1999) as well as IAA and IBA (*amt1*; Ludwig-Müller et al., 1993). Although it can be hypothesized that higher levels of the precursor IAA could increase the rate of IBA synthesis, this was not the case. The *RTY1/SUR1* gene was identified as the *C–S* lyase in glucosinolate biosynthesis and may cause a 'high-auxin' phenotype by accumulation of endogenous *C–S* lyase substrates as well as aldoximes, including indole-3-acetaldoxime which is channeled into IAA (Mikkelsen et al., 2004). In addition, an aldehyde oxidase with strong substrate preference for indole-3-acetaldehyde was about five times higher expressed in *sur1* than in wild type plants (Seo et al., 1998) also probably adding to the elevated auxin levels. *sur1* seedlings showed decreased IBA synthetase activity compared to the wild type. Interestingly, the *trp1-100* mutant defective in phosphoribosyl-anthranilate transferase (Rose et al., 1997) showed also a decrease in IBA synthetase activity compared to wild type. The second mutant with elevated auxin levels *amt1* did not show any differences in IBA synthetase activity compared to the wild type. *AMT1* codes for the α-subunit of anthanilate synthase (Kreps et al., 1996). High levels of IAA may therefore direct the flux towards IAA conjugates and not to IBA. In this context it is noteworthy that IAA itself does not induce IBA synthetase activity (Ludwig-Müller et al., 1995b).

A transgenic line with elevated levels of IAA conjugates, especially IAA-glucose (Ludwig-Müller et al., 2005b) possess higher IBA synthetase activity. Since the increase in IBA synthetase activity was only observed in one of the two trangenic lines tested, the effect may be unrelated to the transgene. Nevertheless, an *Arabidopsis* line with high levels of IBA synthetase activity is a useful tool for further purification of the enzyme.

The increase in IBA synthetase activity in *aux1* may be attributed to the alterations caused by inhibition of the auxin influx facilitator and thus subsequent alterations of cellular auxin distribution. It has yet to be shown whether *rib1* is a component of the auxin transport machinery, although some experiments support this hypothesis (Poupart and Waddell, 2000). This mutant however, showed a decrease in IBA synthetase activity compared to the wild type. Although the levels of IAA and IBA were not uniform for the different growth conditions, the conditions under which the *rib1* seedlings were grown for the enzyme assay may reflect probably better the growth situation on agar, so the slight decrease in IAA as substrate for IBA synthetase may be the cause for reduced activity in the mutant. However, it cannot be conclude that alterations in IBA synthetase activity would be directly reflected in IBA levels, because the scenario might be much more complex involving also IAA and its conjugates (see above).

Other hormones possibly involved in the regulation of IBA synthesis

HLS1 encodes a *N*-acetyltransferase (Lehman et al., 1996) and mutant *hls1* plants failed to display the apical hook in the absence of ethylene which coincides with a reduced ethylene production (Guzman and Ecker, 1990). HLS1 is obviously not directly involved in IBA synthesis, since the mutant phenotype did not correlate with lower IBA synthetase activity. On the contrary, the activity was higher in this mutant compared to Col. Since it was reported that the *HLS1* transcript was inducible by ethylene (Lehman et al., 1996), IBA synthetase activity was investigated in the ethylene overproducing mutant *eto2* (Vogel et al., 1998). The mutation confers higher stability to one of the 1-aminocyclopropane carboxylic acid synthase genes (ACS5; Chae et al., 2003). In *eto2* seedlings, lower IBA synthetase activity compared to wild type was measured. If indeed the absence of the HLS1 protein would cause an increase in IBA synthesis, then an increase in HLS1 by ethylene should result in the observed decrease in IBA synthetase activity.

During progressive drought stress prominent changes in the root morphology of maize seedlings were observed which coincided with differences in IBA synthetase activity (Ludwig-Müller et al., 1995b). While drought stress induced IBA synthesis, seedlings cultured under hypoxia showed less IBA synthetase activity. Drought stress was able to increase the endogenous content of ABA and exogenous ABA was able to induce IBA synthetase activity in maize (Ludwig-Müller et al., 1995b). *Arabidopsis* also responded to drought stress, although different ecotypes showed a different degree of response on two substrates (Figs. 2 and 3). This indicated probably different mechanisms to deal with stress factors. To better define stress conditions, further investigations should be carried out including drought stress-inducible markers.

Drought rhizogenesis is an adaptive strategy that occurs during progressive drought stress and is characterized in *Arabidopsis* and other Brassicaceae and related families by the formation of short tuberized hairless roots (Vartanian et al., 1994). These roots are capable of withstanding a prolonged drought period and give rise to a new functional root system upon rehydration. This response was dramatically reduced in the ABA deficient mutant *aba1*, the ABA-insensitive mutant

abi1, and the auxin-resistant mutant *axr1* (Vartanian et al., 1994).

An increase of IBA synthetase activity in *aba1* seedlings when cultivated under drought stress was observed (data not shown). This effect was specific because the mutants *amt1* and *aux1* did not show a similar response. Based on the results obtained in maize, where drought increased ABA levels, one would hypothesize that in *aba1*, which is defective in zeaxanthin epoxidase (Rock and Zeevaart, 1991), the response to ABA would be diminished. The *aba3* mutation is in the MoCo sulfurase catalyzing the oxidation step from abscisic aldehyde to ABA (Xiong et al., 2001). Both ABA-deficient mutants showed increased IBA synthetase activity under control conditions despite their decreased capacity to synthesize ABA. Maybe the ABA exogenously applied and the stress-induced ABA constitute a different pool which can induce IBA synthetase activity.

Photomorphogenesis

The reaction from IAA to IBA in maize may be a two-step reaction, where an intermediate was released exclusively in the dark (Ludwig-Müller et al., 1995a). Conversely, microsomal membranes from light-grown seedlings synthesized IBA without releasing an intermediate. Photomorphogenetic mutant such as the *det/cop/fus* mutants may be valuable tools to investigate the effect of light on IBA synthetase activity. The *det* mutants show a de-etiolated phenotype in the dark (short hypocotyl, expanded leaves, chloroplast development etc.) The gene product of *DET2* encodes a functional homolog of mammalian steroid 5α-reductases (Li et al., 1996), that catalyzes the conversion of campesterol to campestanol in plant brassinosteroid biosynthesis. The gene product of *DET1* is a nuclear localized protein probably involved in chloroplast differentiation and derepression of chloroplast genes (Chory and Peto, 1990). The *DET3* gene encodes subunit C of the vacuolar H(+)-ATPase (V-ATPase) (Schumacher et al., 1999). Epistasis studies suggested that *DET3* act downstream from *DET1* in the phototransduction pathway (Li et al., 1994). In addition, *det1* showed organ-specific defects in cell elongation and a reduced response to brassinosteroids (BRs). The hypocotyl elongation defect in the *det3* mutant was conditional and evidence was provided that this was due to an alternative mechanism of V-ATPase assembly. Since there is no obvious link to IBA synthesis, the effect of the mutation might be due to unknown regulatory effects.

Root architecture

Root architecture mutants (*cob1*, *cob2*, *scr1*) behaved completely different concerning their ability to synthesize IBA. While *cob1* seedlings showed an increased level of IBA synthetase activity, almost no activity could be detected in *cob2*. Both are semidominant mutations in the same gene, but while *cob1* was selected from an EMS-mutagenized population, *cob2* derived from X-ray mutagenesis (Hauser et al., 1995). *scr1* mutants which have defects in the radial patterning during root development (Wysocka-Diller et al., 2000), did not show any difference compared to the respective wild type. The *cobra* (*cob*) mutants showed a noticeable larger diameter in their roots which was due to abnormal root cell expansion (Benfey et al., 1993; Hauser et al., 1995). The *COB* gene encodes a putative glycosylphosphatidylinositol-anchored protein localized primarily in the plasma membrane of the longitudinal sides of root cells (Schindelman et al., 2001) and was recently annotated as putative phytochelatin synthetase (www.tigr.org). How this gene product could control IBA synthesis has yet to be revealed.

Conclusion

In conclusion, the biosynthesis of IBA might be influenced at several points. The availability of the precursor should play an important role, which is impaired in *amt1* and *trp1-100*, which are involved in anthranilate/tryptophan synthesis, as well as in *sur1* which is involved in glucosinolate and IAA biosynthesis. The lines WD8 and WD15 overexpress showing IAA glucose synthase from maize, but despite both lines showed elevated levels of IAA-glucose, their physiological response regarding IBA synthetase activity was not uniform. Elevated ethylene levels in *eto2* may regulate *hls1* because *HLS1* can be induced by ethylene treatment. The photomorphogenetic mutants *det1*, *det2*, and *det3* may be involved in the regulation of IBA synthetase activity in the light or darkness. Mutants defective in ABA synthesis (*aba1*, *aba3*) may be involved in the regulation of IBA during drought and osmotic stress, whereas *aux1* and *rib1* could be involved in the distribution of auxin by transport, although it is not clear whether RIB1 is really a component of auxin transport. Finally, root architectural mutants may have an effect on the regulation of IBA synthesis during root development. It is clear that there are many more mutants or transgenic lines available which might be linked to auxin homeostasis and which could be tested for IBA

synthetase activity. The *Arabidopsis* mutants tested here for their ability to synthesize IBA under different growth conditions may be valuable tools for further studies on the regulation of IBA synthesis and can also be used to purify the enzyme from lines showing high enzyme activity.

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Indole-3-butyric acid in *Arabidopsis thaliana* *III.* In vivo *biosynthesis*

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Abstract

Indole-3-butyric acid (IBA) was identified by HPLC and GC-MS as one of the reaction products after incubation of sterile cultures of *Arabidopsis thaliana* seedlings with labeled indole-3-acetic acid (IAA). This is the first demonstration of IBA biosynthesis in a dicotyledonous plant. After 1 h of incubation most of the IBA was found in the free form, while after longer periods of incubation most of it was detected in conjugated forms. Formation of IBA conjugates was inhibited by the addition of unlabeled IBA. The biosynthesis of IBA and its conjugates was followed throughout the development of the seedlings and at different pH values. All parts of the plant (isolated roots, leaves, shoots and flowers) were able to convert IAA to IBA to the same extent.

IAA was more readily transported than IBA in mature *Arabidopsis* plants. Feeding of labeled phenylacetic acid (PAA) and α-naphthylacetic acid (NAA) to *Arabidopsis* seedlings resulted in a new small peak which was hydrolyzed by 7N NaOH, but the formation of compounds with longer side chains (analogous to IBA) could not be detected.

Abbreviations: IAA = indole-3-acetic acid; IBA = indole-3-butyric acid; NAA = α-naphthylacetic acid; PAA = phenylacetic acid

1. Introduction

Indole-3-butyric acid (IBA) was found to be a naturally occurring plant growth regulator [7, 10]. In recent years several attempts have been made to understand the role of IBA in the rooting process in plants at the metabolic level. Indole-3-acetic acid (IAA) conjugates were found in every plant which was studied [3]. Similarly, IBA was found to be metabolized to conjugates by various plant tissues. IBA-aspartate was identified in mung bean [22] and pea [16], and there is evidence for a peptide conjugate in mung bean [22]. There is proof of at least one ester conjugate which is probably IBA glucose [6, 8]. Plüss et al. [17], Merckelbach et al. [14] and Riov [18] suggested that IAA conjugates, and not IBA conjugates, are subjected to oxidation, and thus deactivation. IBA was also found to be converted to IAA in plants such as olives and grapevines [5] and pear [1].

In 1991 Ludwig-Müller and Epstein [10] reported for the first time the conversion of IAA to IBA in corn seedlings. Recently [13] IBA was identified by HPLC and GC-MS as an endogenous compound in plantlets of the crucifer *Arabidopsis thaliana* (L.) Heynh. It was demonstrated [12, 13] that IAA was present in *Arabidopsis* at higher concentrations than was IBA, but both hormones were affected in the same way by plant age, light intensity, medium pH and flask volume. *Arabidopsis* seedlings metabolized IBA to two major metabolites which were identified tentatively as IBA-glucose and an IBA amide

conjugate [12]. We report here on the factors affecting the *in vivo* biosynthesis of IBA in *Arabidopsis*.

2. Materials and methods

2.1 Radiochemicals

[1-^{14}C]-IAA, 2.2 GBq.mmol^{-1} Sigma; [5-^{3}H]-IBA, 0.268 TBq mmol^{-1}, Nuclear Research Center, Dimona, Israel; [2-^{14}C]-IAA, 1.99 GBq mmol^{-1}, Amersham, UK; [5-^{3}H]-IAA, 803 GBq mmol^{-1} from Amersham, UK, [1-^{14}C]-phenylacetic acid (PAA), 1.25 GBq.mmol^{-1}, Sigma, and [1-^{14}C]-naphthylacetic acid (NAA), 2.89 GBq.mmol^{-1}, Sigma.

2.2 Plant material

Arabidopsis thaliana (L). Heynh. Columbia ecotype plants were grown from seed in liquid shaking culture using MS medium [15] under continuous illumination as described previously [13]. The pH value of the medium was routinely 5.8 except for the pH experiments, where the pH was brought to levels from 4.0 to 8.0 one day before harvest. Five-day-old seedlings were used routinely for the experiments, except for the developmental experiments, in which they were harvested between 3 and 11 days of age.

For the generation of mature plants, seeds were surface sterilized and placed on agar MS medium. The plants were grown under continuous illumination as described above until flowering. Single plants were removed from the agar and placed in vials containing 5 kBq [1-^{14}C]-IAA or 10 kBq [5-^{3}H]-IBA. After 1 h of incubation at pH 6.0 the plants were removed from the vial, washed, and separated into roots, leaves, the lower and upper parts of the shoots, and flowers. The tissues were homogenized as described above. Each tissue was also sliced into 2-mm segments before it was incubated with 50 m*M* MES buffer (Sigma), pH 6.0, containing labeled IAA. These experiments were conducted to exclude transport phenomena. After 1 h of incubation the tissue was removed and homogenized as above.

2.3 Feeding of radioactive substrates and extraction of labeled compounds

Between 0.5 to 5 kBq of [1-^{14}C]-IAA, [2-^{14}C]-IAA, [^{3}H]-IAA, [^{14}C]-phenylacetic acid, and [^{14}C]-naphthylacetic acid was added to 100 ml Erlenmeyer flasks of 3–11 day old cultures of *A. thaliana* under sterile conditions, and the cultures were then incubated for periods of 30 min to 48 h. The experiments were conducted at pH 5.0. After various periods of incubation the tissue was filtered and washed several times with distilled water. The seedlings were ground with a mortar and pestle with 70% acetone and the extract was centrifuged for 10 min at 15 000 g. Aliquots were removed from the extract to determine the uptake of the labeled substrate into the tissue. The supernatant was centrifuged, evaporated to dryness under a stream of nitrogen, and resuspended in 100 μl methanol. To confirm that IAA was converted to IBA, we fed [$^{13}C_6$]-IAA to cultures of 5-day-old seedlings of *Arabidopsis* in 100 ml flasks. Five hundred ng of [$^{13}C_6$]-IAA was added to each culture (20 cultures in all). After 1 h of incubation the plant material was harvested, extracted and purified for GC-MS identification.

2.4 Alkaline hydrolysis

Hydrolysis of conjugated auxins was performed with 1*N* NaOH at room temperature for 1 h or with 7*N* NaOH at 100 °C under N_2 for 3 h. The hydrolysate was filtered, the pH was brought to 3.0, and the auxins were extracted with equal volumes of ethyl acetate. The ethyl acetate fraction was evaporated to dryness and the sample was resuspended in 100 μl methanol.

2.5 HPLC analysis

HPLC was performed using a reversed phase C_{18} column, the solvents (1% acetic acid in water [A] and methanol [B]) were administered as a linear gradient from 30% to 60% B (0–20 min), and elution was continued for an additional 10 min with 60% B. Flow rate was 0.7 ml·min^{-1}, fractions were collected every 1 min and radioactivity was counted. The detector was set at 280 nm for the detection of IAA and IBA and at 220 nm for PAA and NAA.

2.6 GC-MS analysis

For GC-MS analysis the peak of Rt 21.0 min (IBA) was collected, evaporated and methylated with diazomethane [2]. GC-MS identification was performed with a Hewlett Packard 5971 A mass

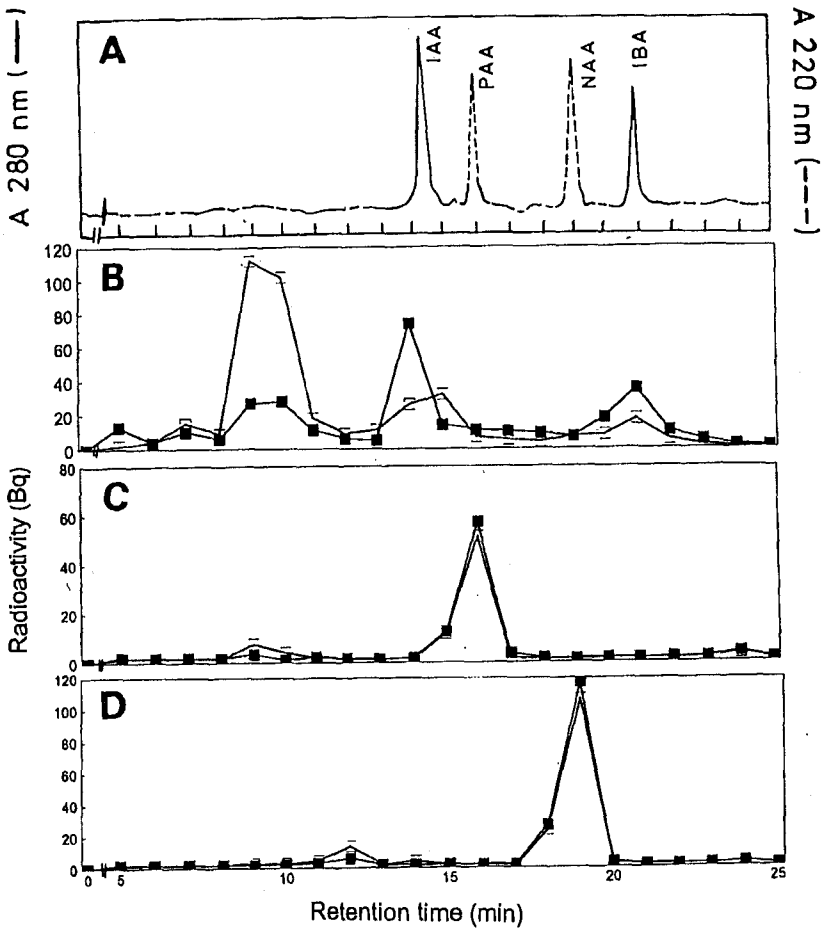

Fig. 1. HPLC separation on reversed phase C_{18} column of extracts of *Arabidopsis thaliana* after incubation with labeled IAA (B), PAA (C), and NAA (D). In (A) the respective standards are presented (Rt IAA = 14.5 min, Rt PAA = 16.1 min, Rt NAA = 19.2 min, Rt IBA = 20.98 min). (□) Without alkaline hydrolysis, (■) after hydrolysis with 7*N* NaOH for 3 h at 100 °C.

selective detector using electron impact ionization. The GC was equipped with a Durabond-5 column, 30 m × 0.25 i.d., 0.25 mm film (J & W Scientific, Folsom, USA). The temperature program was 140 °C for 2 min, followed by an increase of 10 °C min^{-1} to 200 °C and of 20 °C min^{-1} to 250 °C. Identification of [$^{13}C_6$]-IBA was carried out by selected ion monitoring (SIM).

2.7 Statistical treatment of the data

All experiments were repeated at least three times with independently cultured plants. For clarity SEM values are not indicated in the figures, but were always ≤ 10%.

3. Results and discussion

3.1 Characterization and identification of the reaction product(s)

HPLC analysis of extracts of sterile seedlings of *Arabidopsis* which were incubated with C-1 labeled IAA showed the formation of two new labeled peaks (Fig. 1). The first peak at Rt 9.5 min probably contained two amide conjugates, since it was hydrolyzed by 7*N* NaOH to IAA (Rt = 14.5 min) and IBA (Rt = 21.0 min). The second labeled peak at Rt 21.0 min was co-chromatographed with an authentic IBA standard. IBA was identified previously as an endogenous compound

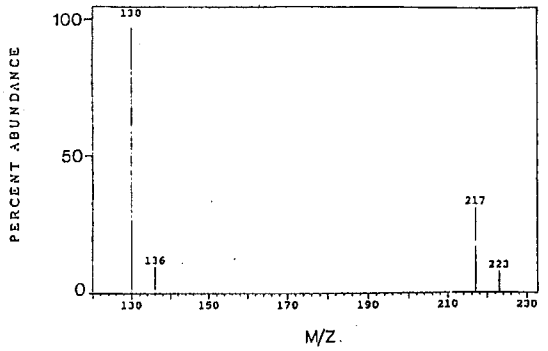

Fig. 2. GC/MS/SIM chromatogram of methylated HPLC purified extract of *Arabidopsis thaliana* after 1 h of incubation with [$^{13}C_6$]-IAA. For identification of [$^{13}C_6$]-IBA the ions 223, 217, 136 and 130 are presented.

in *A. thaliana* [13]. The biosynthesis of this auxin was also examined in maize seedlings [10] and found to be dependent on IAA as a precursor.

HPLC of *Arabidopsis* extracts after incubation with labeled PAA and NAA showed the formation of new small peaks at Rt 9.0 and Rt 12.2 min, respectively (Fig. 1 C, D), which were hydrolyzed by 7*N* NaOH. No significant amounts of phenylbutyric acid and naphthalenebutyric acid could be detected. Smaller amounts of PAA and NAA than of IAA were taken in by the seedlings (Table 1). The transport of various auxins in *Arabidopsis* is currently under study in our laboratory and will be reported in a separate paper. Feeding of IAA labeled in different positions showed that the C-1 (carboxy) and C-2 (methylene), as well as the ring moiety were retained during biosynthesis of IBA (Table 1). In all cases 12–15% of the total radioactivity taken into the seedlings was converted to IBA after 1 h.

Feeding of [$^{13}C_6$]-IAA to *Arabidopsis* seedlings resulted in the formation of [$^{13}C_6$]-IBA, as was demonstrated by SIM (Fig. 2). This was previously reported by Ludwig-Müller and Epstein [10] in corn. They proposed that the *in vitro* biosynthesis of IBA from IAA is carried out by a mechanism similar to that of fatty acid biosynthesis [21]. *In vitro* experiments with microsomal membranes from dark grown corn detected a labeled product after incubation of [^{14}C]-IAA with acetyl-CoA or propionyl-CoA as cofactors. Their formation was increased by adding ATP or Mg^{2+}, respectively [11] and the reaction product was stained positive to indole with Ehmann's reagent [4]. This product was converted to IBA by an organelle fraction (obtained after centrifugation at 10,000g) prepared from the same organism [11]. Further attempts to identify this unknown reaction product by NMR and GC-MS failed due to the small quantity available (J. Ludwig-Müller, C. Griesinger and E. Epstein, unpublished results).

3.2 Some characteristics of IBA formation in seedlings

A time course study of the conversion of [^{14}C]-IAA to IBA (Fig. 3) showed the formation of a labeled IBA peak (34 Bq, or 14% of the radioactivity taken up) after 1 h of incubation. After longer periods of incubation the peak decreased to a constant level of ca 5% of the radioactivity taken up, and new peaks appeared, probably conjugates of IAA and IBA. After 24 h, IAA and IBA conjugates reached a level of 978 Bq (ca 50%) and 498 Bq (28%), respectively. The values given in percentage represent the ethyl acetate extractable amount of radioactivity at pH 3.0 at different incubation times. Uptake of labeled IAA after 1 h showed that ca 240 Bq were extractable from the tissue, whereas the amount of radioactivity extractable after 24 h increased about 7.5-fold up to ca

Table 1. Uptake of IAA, labeled at various positions, [1-^{14}C]-PAA and [1-^{14}C]-NAA and synthesis of IBA after 1 h of incubation with 5-day-old seedlings of *Arabidopsis thaliana*.
Radioactivity of IBA was determined after HPLC on a reversed phase C_{18} column

Isotope	Radioactivity (Bq)	Uptake (Bq)	Uptake (%)	IBA (Bq)	IBA (%)
[1-C^{14}]-IAA	4252 ± 98	944 ± 52	22	129 ± 7	14
[2-C^{14}]-IAA	751 ± 24	145 ± 9	19	18 ± 0.8	12
Ring-^{3}H-IAA	474 ± 16	76 ± 5	16	22 ± 1.3	15
[1-C^{14}]-PAA	3839 ± 103	184 ± 14	5	–	–
[1-C^{14}]-NAA	5525 ± 178	232 ± 20	4	–	–

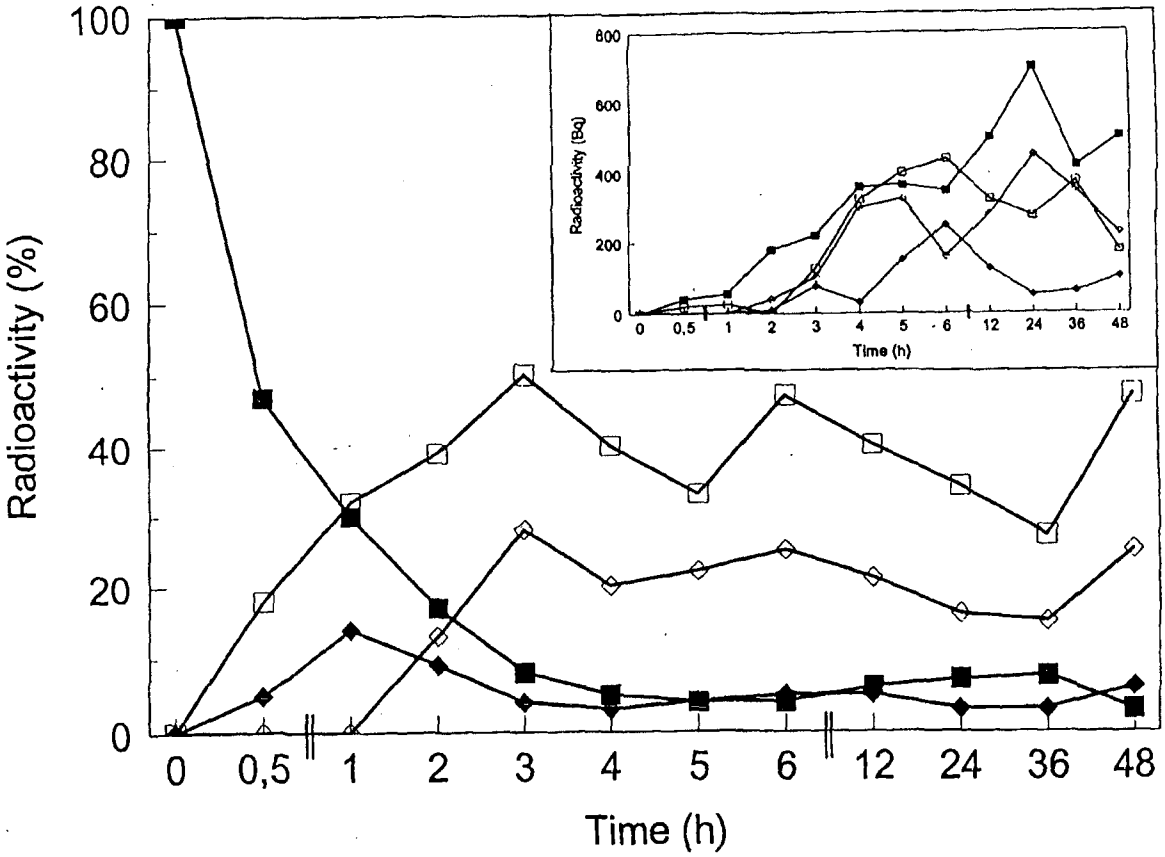

Fig. 3. Time course study of the conversion of IAA to IBA and the respective conjugates in 5-day-old seedlings of *Arabidopsis thaliana*. Values are given as percentage of the labeled IAA taken up. (■) Free IAA, (□) conjugated IAA, (◆) free IBA, (◇) conjugated IBA. In the inset the conjugates are shown according to their chemical bond. (□) Ester bound IAA conjugates, (■) amide bound IAA conjugates, (◇) ester bound IBA conjugates, (◆) amide bound IBA conjugates. The values are given as radioactivity (Bq). SEM never exceeded 10%.

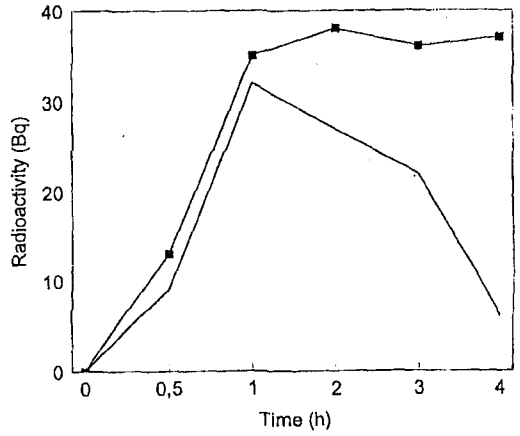

Fig. 4. Time course study of the conversion of IAA to IBA by 5-day-old seedlings of *Arabidopsis thaliana* after the addition of 1×10^{-5}M of unlabeled IBA. (□) [^{14}C]-IAA, (■) [^{14}C]-IAA + unlabeled IBA.

1800 Bq (data not shown). The curves of both auxins followed the same pattern. The inset in Figure 3 (given as total radioactivity) shows the distribution of labeled ester- and amide-bound conjugates of IAA and IBA. IAA was found mainly as an amide conjugate and IBA as an ester conjugate. Determination of endogenous IAA and IBA and their respective conjugates in *Arabidopsis* gave similar results [13]. Autofluorography of extracts of *Arabidopsis* after incubation with [^{3}H]-IBA also showed that an ester conjugate with a high level of radioactivity appeared first, followed by an amide-linked conjugate with lower intensity [12]. Figure 3 also demonstrates an increase in the label of ester-linked IBA corresponding to a decrease in amide-linked IBA, and *vice versa*, suggesting an interrelationship between the two compounds.

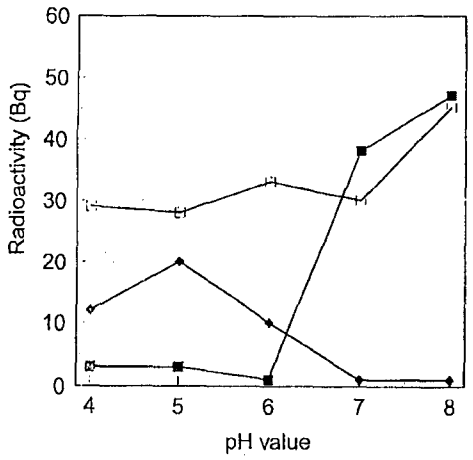

Fig. 5. Formation of free and conjugated IBA after 1 h of incubation with [^{14}C]-IAA at different pH values by 5-day-old seedlings of *Arabidopsis thaliana*. (□) free IBA (■) ester bound IBA, (◆) amide bound IBA.

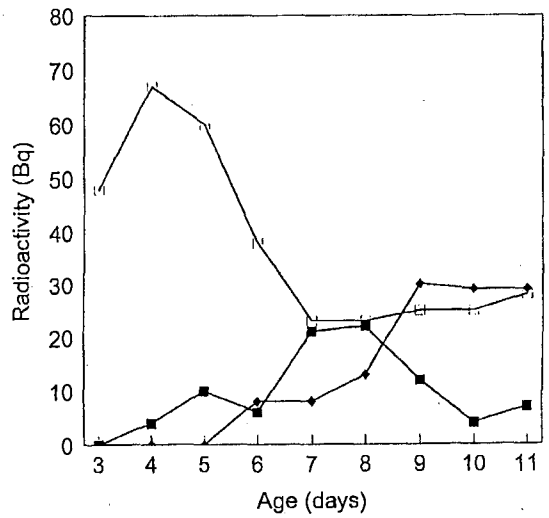

Fig. 6. IBA formation during the first 11 days of development of *Arabidopsis thaliana* seedlings. Incubation time with [^{14}C]-IAA was 1 h. (□) free IBA, (◆) ester-bound IBA, (■) amide-bound IBA.

Addition of 1×10^{-5} M of unlabeled IBA simultaneously with [^{14}C]-IAA brought about the formation of more labeled IBA and there was no decrease in its formation after 1 h of incubation (Fig. 4). The amount of labeled IBA reached a plateau after 2 h of incubation, whereas the amount of free IBA formed without addition of cold IBA decreased after 1 h. This shows that *de novo* synthesis of labeled IBA conjugates (from [^{14}C]-IAA via IBA) was inhibited by the addition of the unlabeled substance.

The *in vivo* biosynthesis of free IBA was not dependent on the pH, and showed only a slight increase at pH 8.0 (Fig. 5). The optimum for the synthesis of ester-bound conjugates was at pH 7.0 and higher, whereas the synthesis of amide-linked conjugates was favoured by a more acid pH (optimum pH 5.0). The pH optimum for IBA biosynthesis in maize seedlings was found to be 6.0–7.0 [10], whereas the optimum for uptake was at pH 6.0. The optimum for IAA-glucose synthase activity in maize was at pH 7.4 [9], and Ludwig-Müller and Epstein [12] found IBA-glucose synthesizing activity in *Arabidopsis* at pH 7.0.

IBA synthesis reached a peak in 3–5 day old seedlings (Fig. 6). Amide-bound conjugates peaked after 7 to 8 days, whereas the ester-bound conjugates increased after the 8th day of development. These data fit very well with the endogenous levels of IBA [13].

3.3. IBA biosynthesis in mature plants

This work with *Arabidopsis* and our preceding work with corn [10] are the first studies to demonstrate the conversion of IAA to IBA. In a similar study by van der Krieken *et al.* [19], IBA biosynthesis could not be detected in cuttings of apple after feeding labeled IAA. This might have been due to longer incubation time, during which most of the free IBA was probably conjugated, as we have demonstrated herein (Fig. 3). It is also possible that in apple cuttings IBA, which was formed from IAA, was converted back to IAA by the tissue. The same investigators reported that not only apple cuttings but also stem slices of apple were not able to synthesize IBA from IAA [20]. They showed that only 2.5% of the exogenous IBA remained in the free state and that the rest was conjugated. 0.4% of absorbed IBA was converted to free IAA. Again, the high potential of those apple slices to convert IBA to its conjugates suggests that also the IBA formed from IAA is rapidly conjugated. To determine whether the ability of plant tissue to convert IAA to IBA is dependent on the plant age, whole mature plants of *Arabidopsis* were incubated with [^{14}C]-IAA and the formation of labeled IBA in various parts of the plant was studied (Fig. 7). Segment 0 repre-

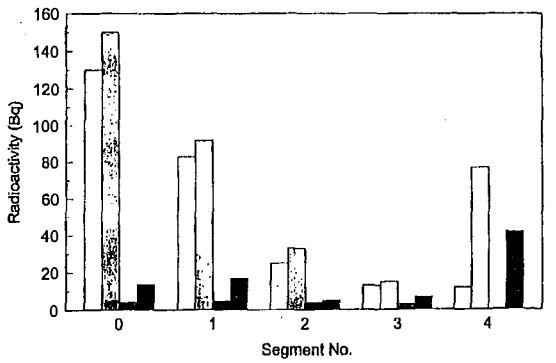

Fig. 7. Distribution of free and conjugated labeled IAA and IBA after feeding of [^{14}C]-IAA to intact mature plants of *Arabidopsis thaliana*. Analysis of metabolites was done after 24 h in different segments. Segment 0: roots; segment I: leaves; segment II: lower part of shoot; segment III: upper (branched) part of shoot; segment IV: flowers. (□) Free IAA (▦) conjugated IAA, (▨) free IBA, (■) conjugated IBA.

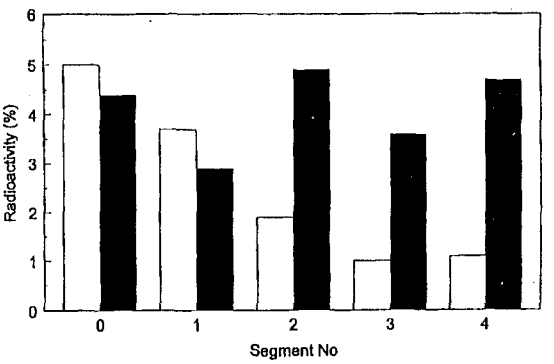

Fig. 8. IBA formation in individual segments of mature *Arabidopsis thaliana* plants. The individual segments (for description see Fig. 7) of 4 mature plants were harvested separately, cut into two mm pieces and incubated with [^{14}C]-IAA for 1 h. (□) IAA uptake as percent of total radioactivity, (■) formation of free IBA as percent of IAA uptake.

sents the roots, segment I the leaf rosette, segments II and III the lower and upper part of the shoot, respectively, and segment IV the emerging flowers. There were still no pods at the time of the experiment. In all segments of the plant, significant amounts of [^{14}C]-IBA or its conjugates, which must have been derived from IAA, were found (Fig. 7). We also noticed differences in the ratio of free: conjugated auxins in the various segments. In the flowers, most of the IAA and all of the IBA were found as conjugates, whereas in all other segments the free form of the two hormones was also present in significant amounts. More conjugated IBA was present in the roots and leaves than in the two shoot segments.

Alternatively, 2-mm sections of the various plant segments were incubated with labeled IAA and rate of uptake and IBA formation was studied (Fig. 8). The rate of IAA uptake was found to differ between the various segments (from 5% in the roots to 1% in the upper shoot and flower segments). The rate of IBA formation varied only slightly (between 3% and 5%) when calculated on the basis of percent radioactivity uptake. It may be concluded that both mature and young *Arabidopsis* plants are able to metabolize IAA to IBA and the respective conjugates of both hormones.

Transport experiments showed that IAA is taken in and transported better than IBA (Fig. 9). No IBA was found in the flowers even after 24 h, and very little label remained in the roots. More IBA

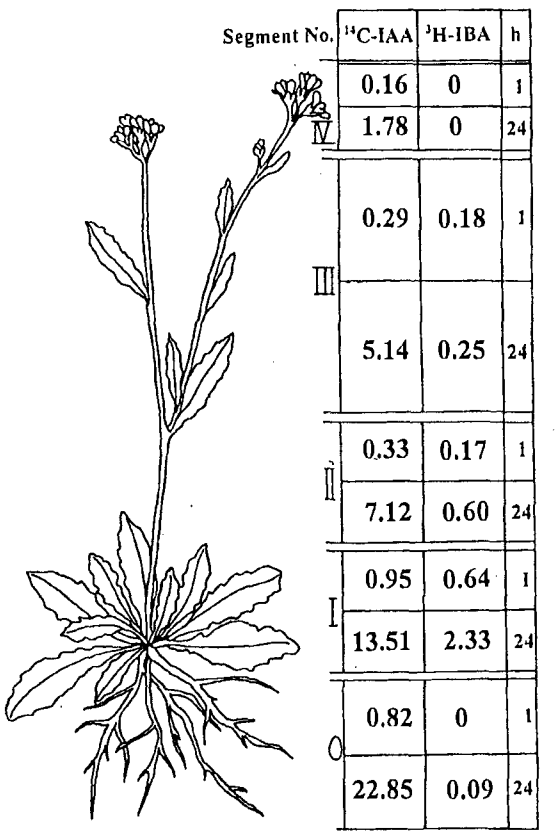

Fig. 9. Uptake and transport of [^{14}C]-IAA and [^{3}H]-IBA in mature plants of *Arabidopsis thaliana* after 1 and 24 h of incubation. For description of the segments, see Figure 7. The values are given as percentage of radioactive IAA and IBA taken in by the plant. The values represent means of four experiments with individual plants.

accumulated in the leaves, whereas most of the IAA was found in the roots. These observations are in contrast to those made by van der Krieken *et al.* [19], who reported that IBA was taken up better than IAA and also was transported better in the shoots.

This is the first demonstration of IBA biosynthesis in a dicotyledonous plant. During our detailed studies we have shown that the main differences between IAA and IBA in *Arabidopsis* seem to be the different paths of conjugation and transport. Further experiments on the regulation of the endogenous concentrations of those two hormones might shed light on the different functions of IAA and IBA during the development of the plant.

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Indole-3-butyric acid in plants: occurrence, synthesis, metabolism and transport

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Abstract

Keywords:
Auxin; auxin conjugates; auxin transport; IAA; IAA aspartic acid; IAA glucose; IBA aspartic acid; IBA glucose; rooting

Indole-3-butyric acid (IBA) was recently identified by GC/MS analysis as an endogenous constituent of various plants. Plant tissues contained 9 ng g^{-1} fresh weight of free IBA and 37 ng g^{-1} fresh weight of total IBA, compared to 26 ng g^{-1} and 52 ng g^{-1} fresh weight of free and total indole-3-acetic acid (IAA), respectively. IBA level was found to increase during plant development, but never reached the leve of IAA. It is generally assumed that the greater ability of IBA as compared with IAA to promote rooting is due to its relatively higher stability. Indeed, the concentrations of IAA and IBA in autoclaved medium were reduced by 40% and 20%, respectively, compared with filter sterilized controls. In liquid medium, IAA was more sensitive than IBA to non-biological degradation. However, in all plant tissues tested, both auxins were found to be metabolized rapidly and conjugated at the same rate with amino acids or sugar.

Studies of auxin transport showed that IAA was transported faster than IBA. The velocities of some of the auxins tested were 7. 5 mm h^{-1} for IAA, 6. 7 mm h^{-1} for naphthaleneacetic acid (NAA) and only 3. 2 mm h^{-1} for IBA. Like IAA, IBA was transported predominantly in a basipetal direction (polar transport). After application of ^{3}H-IBA to cuttings of various plants, most of the label remained in the bases of the cuttings. Easy-to-root cultivars were found to absorb more of the auxin and transport more of it to the leaves.

It has been postulated that easy-to-root, as opposed to the difficult-to-root cultivars, have the ability to hydrolyze auxin conjugates at the appropriate time to release free auxin which may promote root initiation. This theory is supported by reports on increased levels of free auxi in the bases of cuttings prior to rooting. The auxin conjugate probably acts as a 'slow-release' hormone in the tissues. Easy-to-root cultivar were also able to convert IBA to IAA which accumulated in the cutting bases prior to rooting. IAA conjugates, but not IBA conjugates, were subject to oxidation, and thus deactivation. The efficiency of the two auxins in root induction therefore seems to depend on the stability of their conjugates. The higher rooting promotion of IBA was also ascribed to the fact that its level remained elevated longer than that of IAA, even though IBA was metabolized in the tissue.

IAA was converted to IBA by seedlings of corn and *Arabidopsis*. The K_m value for IBA formation was low (approximately 20 μ*M*), indicating high affinity for the substrate. That means that small amounts of IAA (only a fraction of the total IAA in the plant tissues) can be converted t IBA. It was suggested that IBA is formed by the acetylation of IAA with acetyl-CoA in the carboxyl position via a biosynthetic pathway analogous to the primary steps of fatty acid biosynthesis, where acetyl moieties are transferred to an acceptor molecule. Incubation of the soluble enzyme fraction from *Arabidopsis* with ^{3}H-IBA, IBA and UDP-glucose resulted in a product that was identified tentatively as IBA glucose (IBGlc). IBGlc was detected only during the first 30 min of incubation, showing that it might be converted rapidly to another conjugate

Plant Growth Regulation 13: 179–187, 1993.

Indole-3-butyric acid in *Arabidopsis thaliana*

I. Identification and quantification

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Key words: *Arabidopsis thaliana*, auxin conjugates, ethylene, indole-3-acetic acid, indole-3-butyric acid

Abstract

Indole-3-butyric acid (IBA) was identified by HPLC and GC-MS as an endogenous compound in plantlets of the crucifer *Arabidopsis thaliana* (L.) Heynh. *A. thaliana* was cultivated under sterile conditions as shaking culture in different liquid media with and without supply of hormones. Free and total IBA and indole-3-acetic acid (IAA) were determined at different stages of development during the culture period as well as in culture media of different initial pH values. The results showed that IAA was present in higher concentrations than IBA, but both hormones seemed to show the same behaviour under the different experimental conditions. Differences were found in the mode of conjugation of the two hormones. While IAA was mostly conjugated via amide bonds, the main IBA conjugates were ester bound. The ethylene concentration derived from the seedlings, when they were grown in flasks of different size, seemed not to influence the auxin content in the same cultures.

1. Introduction

In horticultural practice natural and synthetic auxins are widely used for stimulating rooting [14, 21]. Many reports have shown that IBA is a better promoter of lateral root formation than IAA [7, 14, 21], and it was suggested that this is due to the higher stability of IBA to oxidation in the plant [14, 21]. In addition, although IBA was long thought to be a synthetic compound, several studies provided evidence for its occurrence as a natural constituent of plants [2, 5, 8–11, 22]. There is evidence for conjugation of IBA via amide linkage in higher plants. Andreae and Good [1] reported that IBA-treated tissues accumulated substances which were tentatively identified as indolebutyramide and indolebutyrylaspartic acid (IBAsp). Chromatographic data provided by Fawcett et al. [12] also indicated the formation of IBAsp from exogenous applied IBA. Wiesmann et al. [24] demonstrated that IBA as well as IAA were rapidly metabolized in mung bean and that conjugation was the major pathway of IAA and IBA metabolism in this tissue. The IBA conjugates were identified as IBAsp and at least two high molecular weight conjugates, where IBA is coupled by an amide linkage [25]. Nordström et al. [22] identified IBA and IBAsp by GC-MS and NMR in pea cuttings during adventitious rooting.

In this report we have examined the occurrence of free and conjugated IBA in sterile cultures of *A. thaliana* following various treatments.

2. Materials and methods

2.1 Plant material

Arabidopsis thaliana (L.) Heynh. Columbia ecotype (wild type and amt-1 mutant [15]) plants were grown from seeds in liquid shaking culture medium (250 ml flasks) under continuous

illumination (0-33 μEinstein cm^{-2}) using either Murashige-Skoog [20], Gamborg [13], or Linsmayer-Skoog [17] medium. The media were adjusted to pH 5.8, except for the pH experiments where it was brought to 4.0-8.0, and determined again on the day of harvest. In some experiments 100 μM of 1-naphthylacetic acid (NAA) was added to the medium. In some experiments 2,6-dihydroxyacetophenone (DHAP), an inhibitor of conjugate formation [16], was added at 2 mM to the medium 16 h before harvest. Seedlings were harvested routinely after 7 days, or between 3 and 11 days for the developmental experiments.

2.2 Extraction of free and total IAA and IBA

After various periods (3 to 11 days) plantlets were removed from the liquid medium by filtration, dried between layers of filter paper and weighed. The auxins were extracted with 70% acetone using an Ultra turrax homogeniser, and stirred with PVP for 2 h at 4 °C. Known amounts (2.5 and 1.0 kBq, respectively) of [5-^{3}H]-IBA (268.25 GBq $mmol^{-1}$, NRC, Dimona, Israel) and [1-^{14}C]-IAA (0.22 GBq $mmol^{-1}$, SIGMA) were added as internal standards for quantification. The extract was then filtered, the filtrate evaporated to the aqueous phase and then centrifuged for 20 min at 50,000 g. The supernatant was divided into two portions for the determination of free and total auxin. The sample for free auxin was adjusted to pH 7.0 and partitioned twice against equal volumes of ethyl acetate. The aqueous phase was then adjusted to pH 3.5 and partitioned again with ethyl acetate. The organic phase was dried over Na_2SO_4, evaporated to dryness, taken up in 1% acetic acid and loaded onto a Water's Co 'Sep Pak', a cartridge containing a C_{18} stationary phase. The cartridge was washed with distilled water and eluted with 100% methanol. Hydrolysis of conjugated auxins was done with 1N NaOH at room temperature for 1 h or with 7N NaOH at 100 °C under N_2 for 3 h. After filtration of the extract, the pH was brought to 7.0 and the purification procedure was continued as described above. Twenty mg seed samples were homogenized with 70% acetone and then treated as described above.

2.3 Identification and quantification of IAA and IBA by HPLC

HPLC was performed using a reversed phase C_{18} column, a UV detector at 280 nm, and a gradient system of (A) 100% methanol and (B) 1% acetic acid in water. Flow rate was 0.7 ml min^{-1}. Quantification was done by calculating the percent recovery of the radioactive auxins.

Identification of IAA and IBA was achieved by co-chromatography with authentic standards (Rt IAA 15.63 min, Rt IBA 22.31 min). All experiments were replicated 3 times, and mean values are presented. SEMs were calculated from at least three independent experiments and were always below 15%. For clarity SEM bars are omitted from the figures. Selected values were confirmed by isotope dilution gas chromatography-mass spectrometry with $^{13}C_6$-IAA (Cambridge Isotope Laboratories) and $^{13}C_1$-IBA [23] as internal standards. For GC-MS analysis the peaks of Rt 15.63 and 22.31 min were collected, evaporated and methylated with diazomethane [6].

2.4 GC-MS analysis

GC-MS identification was performed with a Hewlett Packard 5971A mass selective detector using electron impact ionization. The GC was equipped with a Durabond-5 column, 30 m × 0.25 i.d., 0.25 mm film (J & W Scientific, Folsom, USA). The temperature program was 140 °C for 2 min, followed by an increase of 10 °C min^{-1} to 200 °C and of 20 °C min^{-1} to 250 °C. Spectra were taken by both continuous and reconstructed ion chromatography scans and by selected ion monitoring. The results of the mass spectra were confirmed by library search.

2.5 Ethylene determination

Ethylene was determined from the culture flasks by removing a gas sample (1 ml) followed by direct injection into a gas chromatograph (Varian 3700) fitted with a flame ionisation detector (FID). Separation was done on a Poropak R column (80–800 mesh), column temperature was 50 °C, detector temperature 130 °C, and injector temperature 90 °C. The determination was performed from 5 different culture flasks and 3-4 samples were analysed from each flask.

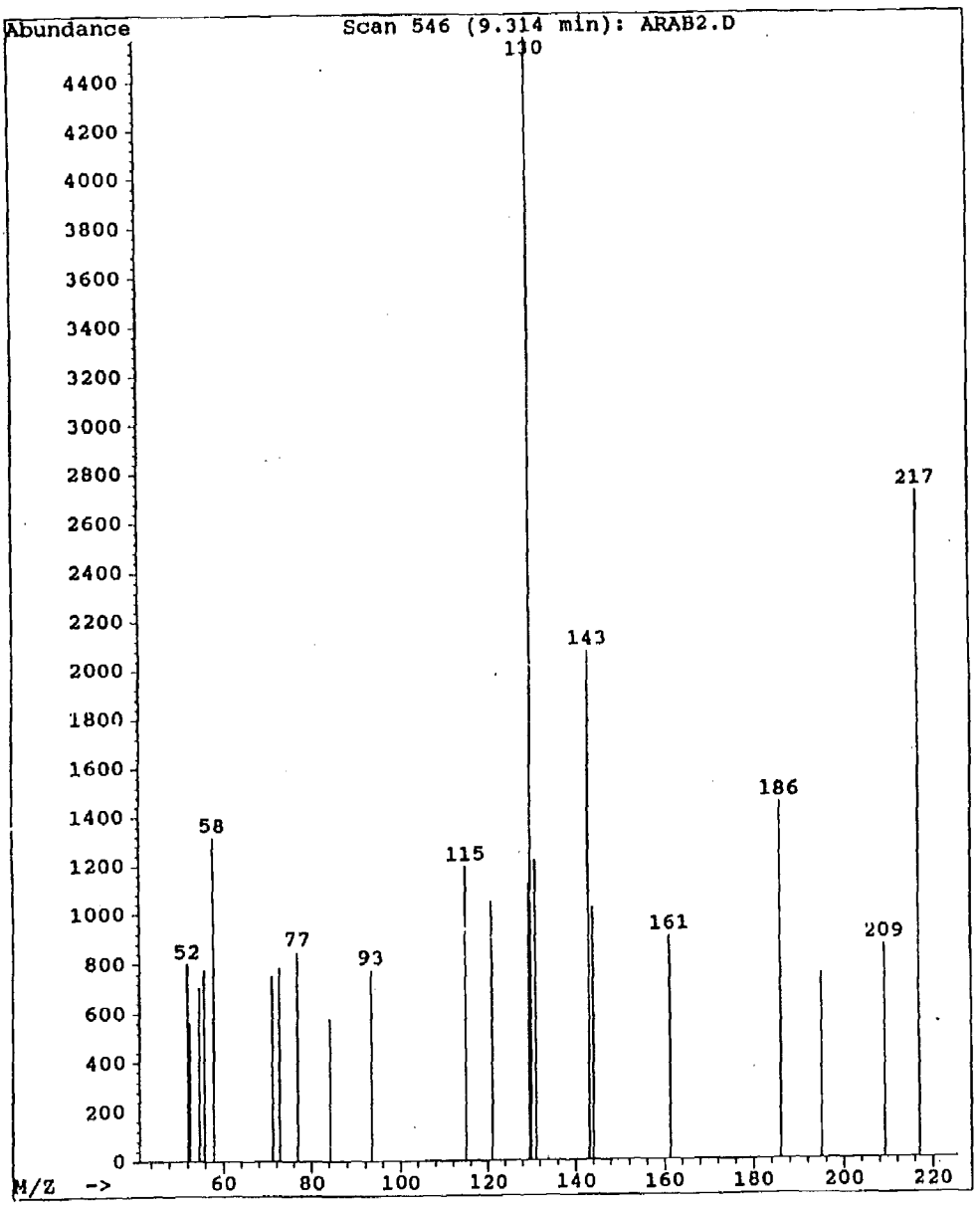

Fig. 1. Total scan mass spectrum of IBA isolated from 7-day-old *Arabidopsis* seedlings.

3. Results and discussion

3.1 Identification of indole-3-butyric acid in Arabidopsis

In the present study the occurrence of indole-3-butyric acid (IBA) has been demonstrated in a cruciferous plant. IBA was identified in seedlings of *Arabidopsis thaliana* by HPLC [19] and GC-MS, where IBA eluted as a single peak at Rt 11.93. Although we have presented a selected ion chromatogram of IBA from *Arabidopsis* earlier [19], Figure 1 shows for the first time the total ion chromatography scan of methylated IBA from *Arabidopsis* at Rt 11.93 with its characteristic fragmentation pattern of 3-substituted indoles. Characteristic ions are at

Table 1. Influence of different media on the content of IAA and IBA after 7 days of culture of *Arabidopsis thaliana* under sterile conditions. SEM was calculated from four independent experiments

Medium	Hormone content (ng g fr wt^{-1})			
	IAA		IBA	
	free	total	free	total
Murashige-Skoog	55 ± 3	920 ± 56	23 ± 2	130 ± 14
Linsmayer-Skoog	42 ± 2	894 ± 47	24 ± 3	119 ± 10
Gamborg	51 ± 5	906 ± 48	19 ± 2	115 ± 12

m/z 217, the molecular ion of methylated IBA, m/z 143 (R–CH=CH$_2$), and the quinolinium ion at m/z 130 [18, 22].

3.2 Quantification of IAA and IBA in Arabidopsis during different culture conditions

All media tested supported growth of *Arabidopsis* with little significant difference in growth. The content of free and bound IBA and IAA in *Arabidopsis* grown on the different media was determined; no significant differences in auxin levels were found (Table 1). The values for free and bound IAA are consistent with others published for dicotyledonous plants [3,4]. Slightly higher values might be the result of the high growth rates of *Arabidopsis* in shaking culture, which would not naturally reflect the situation in mature plants. In tobacco, IBA determinations using ^{13}C-IBA as an internal standard [23] revealed 9 and 37 ng IBA per g fresh weight for free and total hormone concentration, respectively. The magnitude of these values was confirmed by our analysis of IBA in *Arabidopsis*. Our first investigation on the IBA content in *Arabidopsis* [19], which was done only by HPLC, showed higher values, which had to be corrected after GC-MS determination. In

Table 2. Influence of addition of 100 μM NAA on the endogenous auxin content at various ages of *Arabidopsis* seedlings. The seedlings were harvested after 7 days. SEM was calculated from four different experiments

Age of seedlings at addition of NAA (days)	Fresh weight per flask (g)	Hormone content (ng g fr wt^{-1})			
		IAA		IBA	
		free	total	free	total
control	2.28 ± 0.18	60 ± 5	930 ± 54	25 ± 2	117 ± 12
6	2.45 ± 0.22	69 ± 7	1228 ± 70	23 ± 2	131 ± 17
4	2.09 ± 0.10	164 ± 15	1124 ± 66	66 ± 6	316 ± 23
0	0.47 ± 0.02	39 ± 4	779 ± 50	19 ± 1	115 ± 11

Table 3. Influence of light intensity on growth and auxin content in *Arabidopsis thaliana*. The seedlings were cultured seven days under continuous illumination or in the dark. SEM was calculated from four different experiments

Light intensity (μmol cm^{-2})	Fresh weight per flask (g)	Hormone content (ng g fr wt^{-1})			
		IAA		IBA	
		free	total	free	total
31	1.7 ± 0.09	56 ± 6	779 ± 53	21 ± 3	113 ± 10
18	1.4 ± 0.06	50 ± 4	756 ± 49	18 ± 1	115 ± 12
3	1.4 ± 0.07	52 ± 6	769 ± 54	27 ± 3	124 ± 17
0	0.5 ± 0.01	183 ± 16	997 ± 86	32 ± 4	180 ± 22

Table 4. Influence of medium pH on the content of auxins in *Arabidopsis*. The pH was altered at the beginning of the culture period and determined again at the end of the experiment (7 days). SEM was calculated from four different experiments

pH at start of experiment	pH after 7 days	Hormone content (ng g fr wt^{-1})			
		IAA		IBA	
		free	total	free	total
4.0	5.1 ± 0.2	55 ± 6	783 ± 56	16 ± 1	119 ± 13
5.0	5.2 ± 0.1	58 ± 7	863 ± 67	13 ± 1	124 ± 16
6.0	5.2 ± 0.1	47 ± 5	820 ± 59	18 ± 2	126 ± 15
7.0	5.2 ± 0.1	48 ± 5	793 ± 56	15 ± 2	119 ± 11
8.0	5.3 ± 0.2	48 ± 6	833 ± 63	7 ± 0.5	129 ± 14

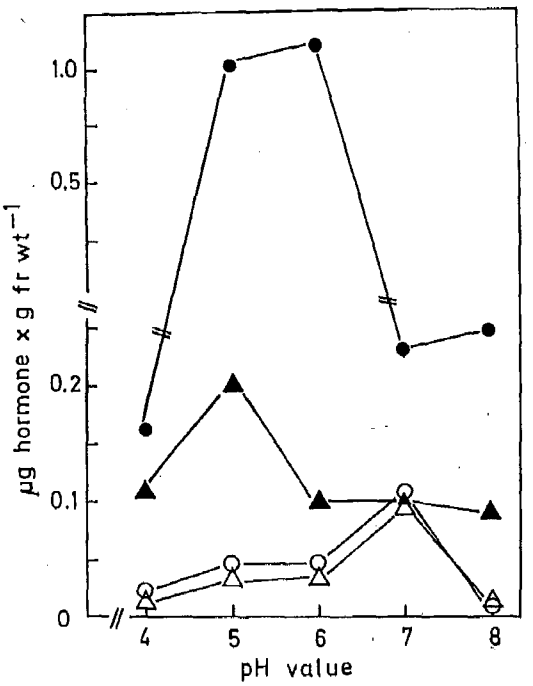

Fig. 2. Free and total IAA and IBA content of *Arabidopsis* seedlings cultured at different pH values. The pH was altered 24 h before harvest. (○) free IAA, (●) total IAA, (△) free IBA, (▲) total IBA.

the present study the differences in concentration between free IAA and IBA were small (ratio 2.5 : 1), whereas the concentrations of the conjugated compounds differed dramatically (8 : 1, IAA : IBA). We also determined the two hormones under different culture conditions to investigate exogenous influences on them.

Addition of 100 μM NAA to the culture medium caused considerable changes in the growth of the seedlings, but was dependent on the time that the hormone was added (Table 2). When added early, NAA inhibited growth and caused an increase in the amounts of conjugated IAA and IBA, probably by a detoxification reaction of the plant. When NAA was added 16 h before harvest, the morphology of the seedlings was not altered and the auxin levels were not significantly changed compared with the control. Similar results were observed after feeding IBA to pea cuttings [22]. Since auxin is not required for the growth of *Arabidopsis* seedlings, they may serve as a better tool than cell suspension cultures for the investigation of the influence of endogenous and exogenous auxins on plant development.

Table 6. Influence of 2 mM 2.6-dihydroxyacetophenone applied to *Arabidopsis* cultures 16 h before harvest compared to non-treated cultures. SEM was calculated from three different experiments

Feature	Control	+2.6-DHAP	% of control
g fresh weight per flask	2.96 ± 0.21	1.26 ± 0.11	43
g dry weight per flask	0.12 ± 0.02	0.09 ± 0.03	75
free IAA ng g fr wt^{-1}	44 ± 5	41 ± 3	95
total IAA ng g fr wt^{-1}	843 ± 68	304 ± 21	36
free IBA ng g fr wt^{-1}	11 ± 0.7	14 ± 1	114
total IBA ng g fr wt^{-1}	134 ± 11	89 ± 7	66

Different light intensities (3–18 μmol cm^{-2}) did not affect the internal hormone content significantly, but high hormone levels were detected after culturing in complete darkness (Table 3), where free IAA increased more than free IBA.

IAA and IBA production in the plant was affected by changing the pH 24 h before harvest. *Arabidopsis* controls the pH of its medium, and therefore longer incubation periods had no influence on the content of the two auxins (Table 4). The pH optima for the formation of the two free

Table 5. Determination of free auxins (IAA and IBA) and ethylene in relation to the volume of the culture flask. SEM was calculated from three different experiments

Flask volume (ml)	fr wt/flask (g)	ethylene nl/flask	ethylene nl/g fr wt	free auxin (ng/g fr wt) IAA	free auxin (ng/g fr wt) IBA
125	2.09 ± 0.18	32 ± 4	15.3 ± 1.3	58 ± 6	23 ± 2
395	3.28 ± 0.23	60 ± 5	18.4 ± 1.7	56 ± 5	22 ± 2
630	3.95 ± 0.64	63 ± 8	17.3 ± 1.5	23 ± 4	14 ± 2
1170	6.80 ± 0.57	216 ± 17	31.8 ± 3.3	46 ± 6	9 ± 1

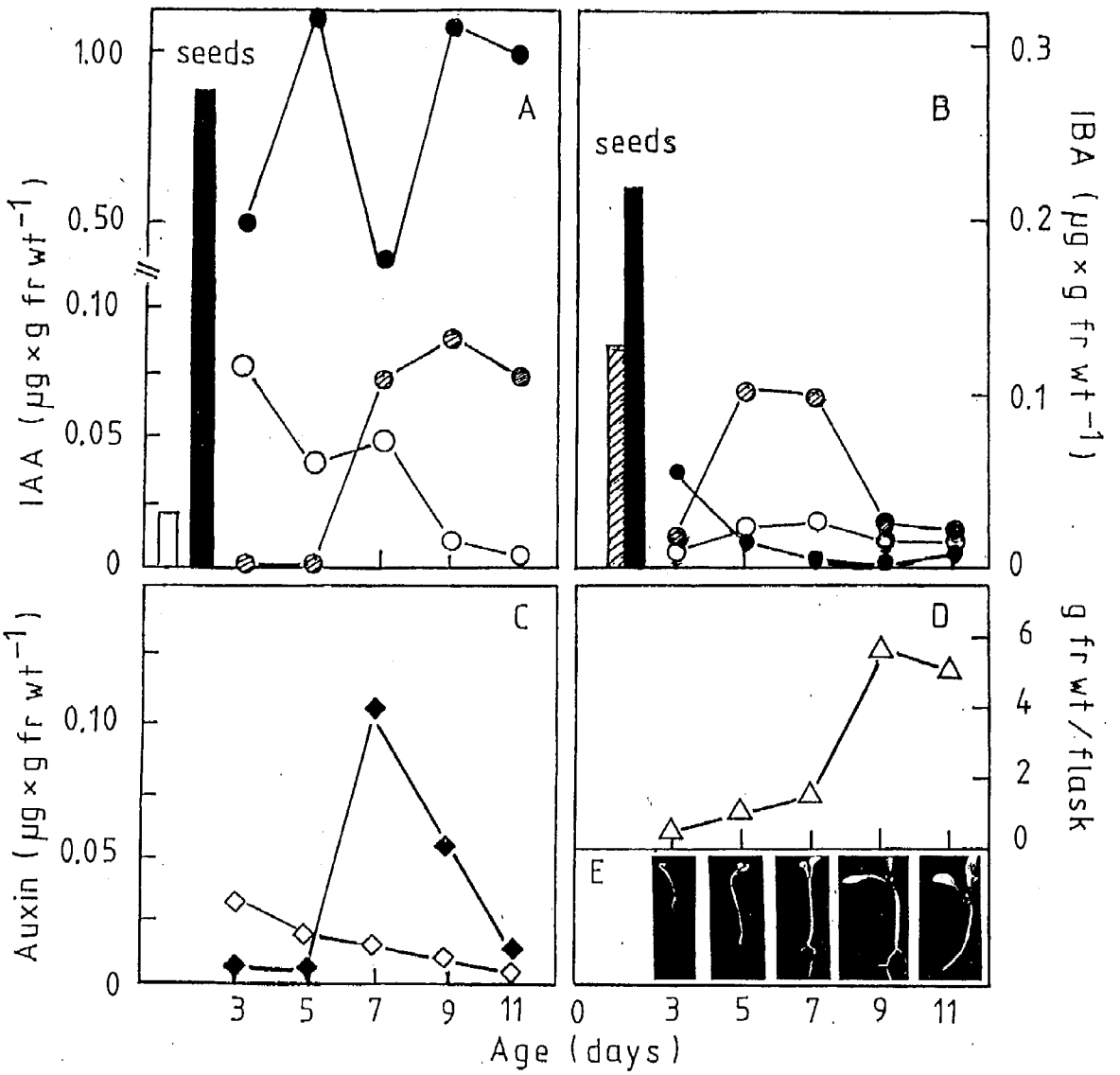

Fig. 3. Free, amide and ester-bound IAA and IBA content during the first days of the development of *Arabidopsis* seedings. A. IAA, (●) amide bound, (◍) ester bound, (○) free hormone; B. IBA, (●) amide bound, (◍) ester bound; (○) free hormone; C. Free IAA and IBA released from *Arabidopsis* seedlings into the medium, (◆) IAA, (◇) IBA; D. Fresh weight of seedlings per flask; E. Morphology of *Arabidopsis* seedlings at the different times of harvest. The histograms in A and B represent IAA and IBA content in the seeds of *Arabidopsis*. (□) free hormone, (▨) ester bound, (■) amide bound.

auxins were 7.0, and for the two conjugated auxins 5.0 (Fig. 2).

The effect of increasing the capacity for gas exchange by the plants were evaluated by changing the size of the culture flasks from 100 ml to 1000 ml. The ratio of medium to flask volume was kept constant and the same number of seeds was added to each flask. Before harvest of the seedlings, 3–4 samples were removed from each flask for ethylene determination. The ethylene content (expressed as nl per flask) increased with the flask volume, but there was no perceptable tendency towards a rise in the ethylene content expressed per g fresh weight, and no correlation between auxin, ethylene and size of culture flask was observed (Table 5).

Table 7. Content of free and conjugated auxin content in wild type plants and a mutant with elevated tryptophan content (amt-1) [15]. SEM was calculated from four different experiments

Plant material	Hormone content (ng g fr wt^{-1})			
	IAA		IBA	
	free	total	free	total
Wild type	58 ± 4	850 ± 76	21 ± 3	112 ± 13
amt-1	72 ± 9	2810 ± 134	74 ± 7	302 ± 25

Treatment with 2,6-dihydroxyacetophenone (DHAP), which was described as a conjugation inhibitor [16], influenced the ratio of fresh weight/dry weight significantly (Table 6), although no visible symptoms were observed in the plants. The differences in weight were reflected in the differences in the content of bound auxins. Free hormone content was not reduced, but total IAA and IBA were decreased by about 64 and 34%, respectively. The differences in the rates of decrease between IAA and IBA might be due to different conjugation mechanisms. In *Teucrium canadense* [16], DHAP is only capable of inhibiting the conjugation via amide bonds. We have shown (see 3.3) that IBA is mostly conjugated via ester bonds, whereas the amide-bound metabolites represent the major fraction of the IAA conjugates.

3.3 IAA and IBA content during the first phase of development

The contents of IBA and IAA were examined during the first days of development of *Arabidopsis* seedlings. The level of free IAA was highest at 3 days, which is a period of high cell growth rate, and then decreased continuously. The increase in IBA level started a little later than IAA and had a maximum at 5–7 days, but did not reach as high a maximum concentration as that of IAA (Fig. 3A+B). Figure 3E shows that the main development of roots occurred during day 5–7, although small roots were visible before. After both strong and mild alkaline hydrolysis conjugated IAA mostly occurred with amide linkage.

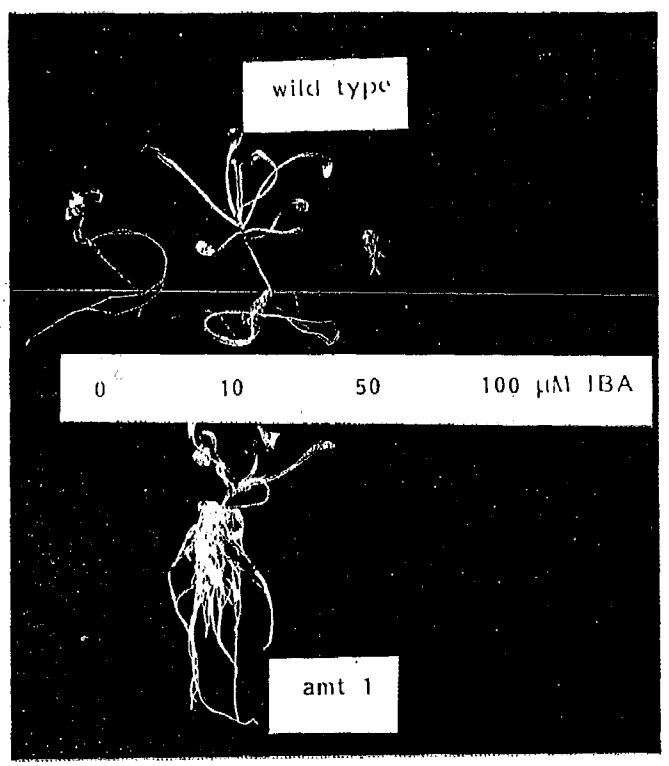

Fig. 4. Effect of 0, 10, 50, and 100 μM IBA on *Arabidopsis* wild type and amt-1 plantlets grown on agar plates with Murashige-Skoog medium.

In the seeds and during the first 5 days no ester-bound IAA conjugates were detected. In contrast, IBA mostly seems to be conjugated via ester bonds and only the seeds show high concentrations of an IBA amide conjugate. This difference in conjugation might be a reason for IBA being a better rooting promoter than IAA, possibly by faster breakdown of the ester conjugates.

During the whole culture period both auxins were released into the medium (Fig. 3C). The free IAA content peaked at 7 days, correlating with low amide-bound IAA (Fig. 3A). Conversely, the IBA content in the medium decreased from day 3 to day 11, following the same pattern as the amide bound conjugate(s). The maximum IBA content was detected, when free and ester bound IBA were lowest in the tissue.

3.4 IAA and IBA in wild type and mutant Arabidopsis plants

High levels of free and conjugated IAA and IBA were found in an *Arabidopsis* mutant with elevated free tryptophan (Table 7). The mutant, amt-1 (resistance to α-methyltryptophan) is described in detail elsewhere [15] and is similar in appearance to the wild type when cultured under normal conditions. When plantlets of wild type and amt-1 were cultivated on agar plates with different IBA concentrations (0–100 μM) they exhibited different behaviour (Fig. 4). Whereas about 50% of the wild type seeds germinated without IBA in the medium, amt-1 seeds did not, as previously described for other auxins [15]. Addition of 10 μM IBA supported growth of seedlings of both mutant and wild type and the amt-1 plants reacted with increased root growth in comparison to the wild type. At 50 and 100 μM IBA, amt-1 seedlings did not grow, whereas 10% of the wild type seedlings per petri dish showed agravitropic root growth and no shoot growth.

The levels of free IAA and IBA were increased in the mutant about 1.2-fold and 3.5-fold, respectively (Table 7). Levels of IAA and IBA were 3.3 and 2.7-fold higher, respectively, after alkaline hydrolysis. The higher auxin levels reflect the higher tryptophan levels (6.8-fold in plants and 2.6-fold in callus, compared to the wild type [15]), and therefore seem to support the role of tryptophan as an auxin precursor under these culture conditions. Interestingly, free IBA content was increased more than IAA in this mutant, which indicates that IBA may serve not only as a storage form of auxin, but also as a detoxification product in some cases. Further studies with such mutants might help to shed light on the actual function of IBA in plants.

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RESEARCH PAPER

Analysis of indole-3-butyric acid-induced adventitious root formation on *Arabidopsis* stem segments

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Abstract

Root induction by auxins is still not well understood at the molecular level. In this study a system has been devised which distinguishes between the two active auxins indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA). IBA, but not IAA, efficiently induced adventitious rooting in *Arabidopsis* stem segments at a concentration of 10 μM. In wild-type plants, roots formed exclusively out of calli at the basal end of the segments. Root formation was inhibited by 10 μM 3,4,5-triiodobenzoic acid (TIBA), an inhibitor of polar auxin transport. At intermediate IBA concentrations (3–10 μM), root induction was less efficient in *trp1*, a tryptophan auxotroph of *Arabidopsis* with a bushy phenotype but no demonstrable reduction in IAA levels. By contrast, two mutants of *Arabidopsis* with measurably higher levels of IAA (*trp2*, *amt1*) show root induction characteristics very similar to the wild type. Using differential display, transcripts specific to the rooting process were identified by devising a protocol that distinguished between callus production only and callus production followed by root initiation. One fragment was identical to the sequence of a putative regulatory subunit B of protein phosphatase 2A. It is suggested that adventitious rooting in *Arabidopsis* stem segments is due to an interaction between endogenous IAA and exogenous IBA. In stem explants, residual endogenous IAA is transported to the basal end of each segment, thereby inducing root formation. In stem segments in which the polar auxin transport is inhibited by TIBA, root formation does not occur.

Key words: Adventitious root formation, *Arabidopsis*, auxin, auxin-inducible proteins, differential display, indole-3-butyric acid, protein phosphatase 2A, TIBA.

Introduction

Root development in *Arabidopsis thaliana* has been the subject of many studies employing mutant screens during the last few years (for a review see Casson and Lindsey, 2003). While development of the primary root from the embryonic stage has received a lot of attention and the processes involved are beginning to unravel, the formation of lateral and adventitious roots is less well understood. Lateral and adventitious roots are formed post-embryonically. While lateral roots typically form from the root pericycle, adventitious roots form naturally from stem tissue. Adventitious roots are less predictable in their cellular site of origin than lateral roots. They may form from the cambium or, in the case of detached stem cuttings, from calli. Therefore it appears that adventitious roots can be formed by two different pathways: (i) direct organogenesis from established cell types or (ii) from callus tissue following mechanical damage (Casson and Lindsey, 2003, and references therein).

Adventitious root formation has many practical implications in horticulture and agronomy and there is a lot of commercial interest because of the many plant species that are difficult to root. (Davies *et al*., 1994; Kovar and Kuchenbuch, 1994). The auxin indole-3-acetic acid (IAA) was the first plant hormone to be used to stimulate rooting of cuttings (Cooper, 1935). At that time it was discovered

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that a second, 'synthetic' auxin indole-3-butyric acid (IBA) also promoted rooting and was even more effective than IAA (Zimmerman and Wilcoxon, 1935). IBA is now used commercially worldwide to root many plant species (Hartmann *et al*., 1990). Since its introduction more than 50 years ago, IBA has been the subject of many experiments, mostly involving trial and error studies to achieve optimum rooting conditions for the plant species in question. Application of IBA to cuttings of many plant species results in the induction of adventitious roots, in many cases more efficiently than IAA (Epstein and Ludwig-Müller, 1993). For example, in *Vigna radiata* the induction of adventitious roots was observed after IBA, but not IAA application (Riov and Yang, 1989). The greater ability of IBA to promote adventitious root formation compared with IAA has been attributed to the higher stability of IBA versus IAA both in solution and in plant tissue (Nordström *et al*., 1991). The effective concentration of IBA in these kinds of studies was also dependent on the pH of the medium. It was shown that, at lower pH values, lower IBA concentrations in the medium were sufficient to induce rooting of apple cuttings (Harbage and Stimart, 1996).

Differences in the ability to form adventitious roots have been attributed to differences in auxin metabolism (Alvarez *et al*., 1989; Blazkova *et al*., 1997; Epstein and Ludwig-Müller, 1993). It was shown, for example, that a difficult-to-root cultivar of *Prunus avium* conjugated IBA more rapidly than an easy-to-root cultivar (Epstein *et al*., 1993). Only in the easy-to-root cultivar was the appearance of free IBA observed after several days and the authors concluded that the difficult-to-root cultivar was not able to hydrolyse IBA conjugates during the appropriate time points of adventitious root development. Interestingly, it was possible to induce rooting of the difficult-to-root cultivar after application of an inhibitor of conjugation (Epstein *et al*., 1993). It has been shown that IBAsp is even more active than free IBA in the promotion of adventitious roots in mung bean, possibly due to its higher stability during the rooting process (Wiesman *et al*., 1989). However, other differences such as uptake and transport can also account for the differences in rooting behaviour (Epstein and Ludwig-Müller, 1993).

The physiological events leading to root initiation may be revealed by using targeted or untargeted molecular approaches to identify genes that may be involved in adventitious rooting. IBA has been identified as a natural substance in *Arabidopsis thaliana* (Ludwig-Müller *et al*., 1993) and there are indications that at least part of the action of IBA is not through IAA in this species (Poupart and Waddell, 2000; Zolman *et al*., 2000). Therefore a system has been devised for adventitious root formation on stems of the model plant *Arabidopsis* under sterile conditions, where roots are specifically induced after the application of IBA but not of IAA. The results have shown that (i) IBA is one important factor in *Arabidopsis* to induce adventitious roots, (ii) the timing of auxin application is important to distinguish between callus and root formation, and (iii) this system is suitable for identifying genes involved in adventitious root formation. Finally, the effect of an auxin transport inhibitor, TIBA, on IBA-induced adventitious root formation has been investigated and IAA-deficient mutants were used to analyse the interplay between IAA and IBA during adventitious rooting.

Materials and methods

Plant material

Arabidopsis plants were grown aseptically on Murashige and Skoog (MS) agar (Murashige and Skoog, 1962) in Magenta® boxes at 24 °C under constant illumination with cool white fluorescent lights, approximately 40 μmol m^{-2}. The seeds were surface sterilized with 5% (v/v) commercial bleach (Clorox; a 5% solution of sodium hypoclorite) for 20 min, washed thoroughly, planted on 1% agar, and vernalized for 24 h at 4 °C. Inflorescences from 4 8 week old plants were used because, during this period, the age of the stems did not influence callus/root formation, although on stem segments of older plants no root formation could be observed; data not shown). The inflorescences were cut into 0.5 cm node free segments and incubated in the dark or under constant illumination in Petri dishes containing full strength MS agar containing the appropriate concentrations of IAA or IBA with or without different concentrations of 3,4,5 triiodobenzoic acid (TIBA). In the light, the plates were covered with yellow plastic to prevent photo oxidation of auxins (Campanella *et al*., 1996). Starting at 5 d, plates were examined daily and the proportion of segments showing callus or root formation was scored.

For the differential display experiments, segment length was reduced to 3 mm to increase the number of ends per fresh weight. For the subsequent treatments, segments were transferred under sterile conditions to fresh Petri dishes containing either plain MS agar or MS agar with the appropriate hormone supplement.

For histology, stem segments were fixed for at least 24 h in FAA (5% formaldehyde, 5% acetic acid, 50% ethanol), then dehydrated through a series of ethanol steps (70%, 80%, 95%) before infiltration with JB 4 resin (Polysciences, Inc., Niles, IL). Sections of 2 4 μm were stained with toluidine blue.

Evaluation of the rooting process

On each Petri dish for the different treatments 10 12 *Arabidopsis* stem segments were placed. Each experimental condition consisted of at least two Petri dishes. All experiments were performed at least three times, resulting in a minimum of 60 segments which were scored per treatment. Mean values of the three independent experi ments are given. After the different treatments the *Arabidopsis* stem segments were inspected for callus or root formation and the number of segments exhibiting the respective organs counted.

RNA extraction and differential display

Isolation of total RNA was performed using TRIzol reagent (Gibco BRL, now marketed by Invitrogen, Carlsbad, CA) according to the manufacturer's instructions using 300 mg fresh weight of treated and control segments. Reverse transcription followed by PCR using anchored VNT_{11} 3′ primers and 10 mer OPA 5′ primers (both Operon Technologies) was performed essentially as described by Liang and Pardee (1992). ^{35}S Radiolabelled amplification products were resolved on 6% acrylamide sequencing gels and detected by autoradiography. The experiment was repeated to show reproducibil ity of fragment induction. Fragments induced only under condition C

were excised, re amplified with the same primer combination, the PCR products purified (QIAquick® gel extraction kit, Qiagen), ligated into pBSK vector, and sequenced from both ends at The Institute for Genomic Research.

Northern blot analysis

Total RNA was isolated as described above. The synthesis of the biotinylated (bio dUTP, Boehringer Mannheim) cDNA probe used for northern hybridization was performed by PCR. Template was cDNA prepared from total RNA of *Arabidopsis* stems induced with IBA. For amplification of the phosphatase 2A like protein subunit as a probe, the following primer pair was designed according to the sequence information obtained: forward 5′ GATCATGTGATA GAAGATAAATTTAGTGCT 3′; reverse 5′ TCTTCTATCACAT GATCTCGTCAGGGACCA 3′. PCR was performed according to standard procedures using the following programme: initial denatur ation at 96 °C for 5 min, followed by 30 cycles of 96 °C for 45 s, 55 °C for 45 s, 72 °C for 45 s. Equal sample loading (20 µg total RNA) was confirmed by hybridization with an *actin 2* (At3g18780) probe amplified with the following primers: forward 5′ GAAGAT TAAGGTCGTTGCACCACCTG 3′; reverse 5′ ATTAACATTG CAAAGAGTTTCAAGGT 3′. Non radioactive northern blots were performed according to Löw and Rausch (1994), with the Northern Light™ kit from Tropix (Serva) for detection.

Results

Indole-3-butyric acid can induce adventitious roots on Arabidopsis *stem segments*

Several reports deal with the better performance of IBA versus IAA during the rooting process. This was attributed to parameters such as stability, transport, or metabolism. Therefore a protocol was devised which would induce adventitious roots on *Arabidopsis* stems by one of the auxins but not the other. This study's experiments showed that several parameters influenced adventitious root induction and helped to discriminate between the actions of IAA and IBA. These were: (i) concentration of the hormone, (ii) duration of treatment, (iii) priming event, and (iv) second hormone treatment.

In a first set of experiments, 0.5 cm explants of *Arabidopsis* stems were incubated for 7 d on MS medium containing either IAA or IBA at different concentrations and the phenotype was recorded (Fig. 1A). Since the explants looked similar when they were cultivated on hormone plates for 7 d, only the explants on different IBA concentrations are shown. The induction of adventitious roots was always preceded by callus formation. Root induction was seen at 1 µM and 10 µM IBA and IAA, and at 100 µM hormone the roots looked stunted with more root hairs produced (Fig. 1A). Similarly, root induction by IAA or IBA was also possible using excised leaves (Fig. 1B). The concentration dependence was also comparable with that for stem segments.

On stem segments treated with IBA, adventitious roots clearly arose from the cambium, which first de-differentiates to form a callus (Fig. 2B). This is followed by the formation

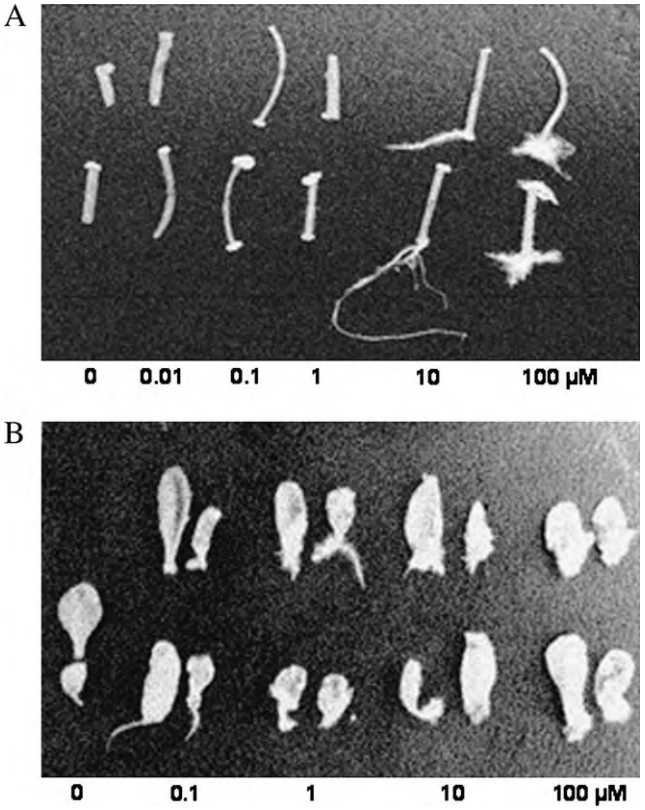

Fig. 1. Root induction on *Arabidopsis* stem (A) and leaf (B) explants after treatment with different IBA concentrations.

of roots (Fig. 2C) that subsequently elongated and by the formation of additional callus areas, which gave rise to new adventitious roots (Fig. 2D). In the controls without IBA such structures were never visible (Fig. 2A).

Timing of hormone requirement for adventitious rooting

To determine the period of IBA exposure required for adventitious root induction, the stem segments were incubated on 10 µM IAA or IBA for different time periods up to 48 h and then transferred to hormone-free MS medium for the remaining time. Callus and root formation was scored at 7 d (Fig. 3). The proportion of explants forming callus increased up to 100% after 48 h on auxin-containing medium (Fig. 3A). While callus formation was comparable on IAA- or IBA-containing MS agar, root formation was found only when IBA was in the medium. After a 6 h exposure, a response was already found, but optimum rooting was observed with a treatment of 48 h (Fig. 3B). After longer incubation periods the difference between IAA and IBA treatment became less pronounced (data not shown). The inset in Fig. 3B shows a picture of stem segments incubated for the respective time on either 10 µM IAA or 10 µM IBA.

A two-stage treatment was developed to distinguish between callus and root induction by IBA (Fig. 4). In stage I,

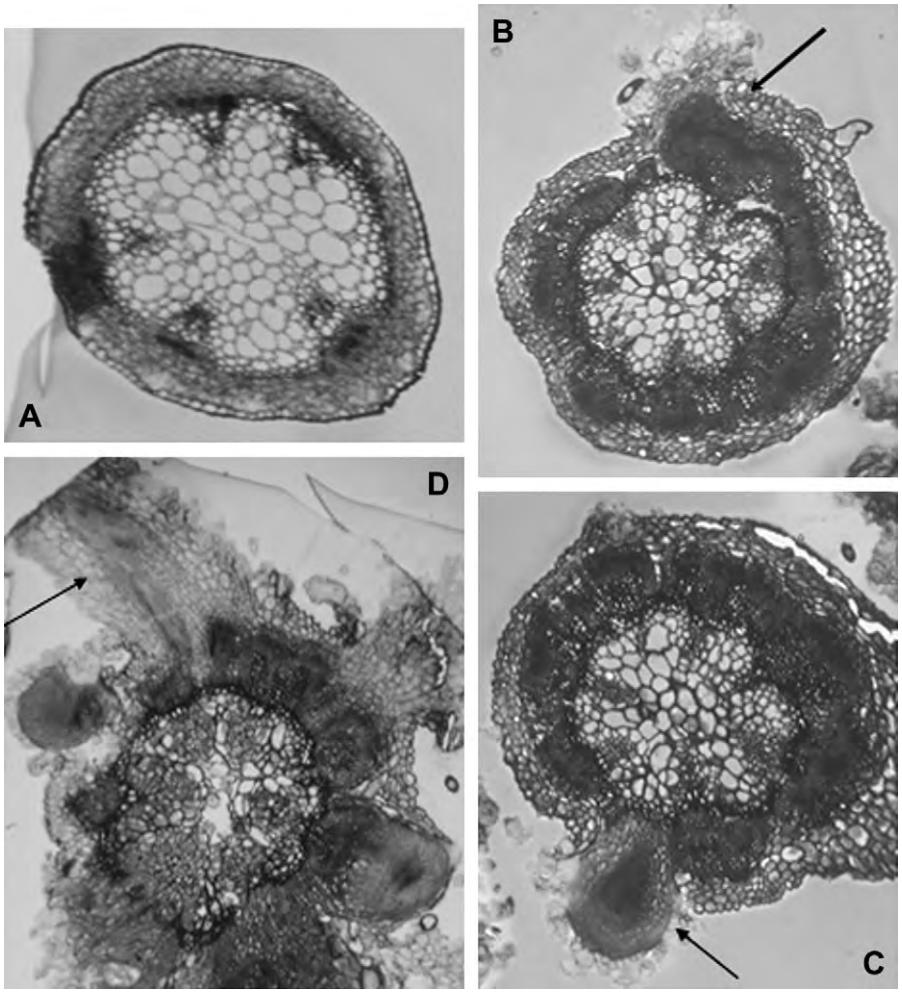

Fig. 2. Development of adventitious roots on *Arabidopsis* stems without (A) and after treatment with 30 μM IBA (B D). Sections were taken 3 d (B), 5 d (C), and 9 d (D) after placing the segments on rooting medium. Sections of 2 4 μm were stained with toluidine blue. Adventitious roots are marked by arrows.

explants were incubated for 24 h on 10 μM IBA, a treatment that resulted in callus formation. In stage III, explants were given a second 10 μM IBA treatment of variable duration after a period of 24 h on hormone-free medium (stage II). The explants were transferred to hormone-free medium after the second IBA treatment for the remainder of the experiment (stage IV) and root formation was scored 14 d after the start of the second treatment. The second treatment resulted in the formation of adventitious roots on 60–95% of the explants, provided that it was at least 48 h long (Figs 4A, 5A). In addition, it was shown that the highest rooting efficiency was found with treatments that involved two exposures to IBA separated by a time without hormone (Fig. 5A). Increasing the incubation time of the second treatment on IBA also resulted in more segments showing adventitious root formation. Interestingly, in the experiments using only one long IBA treatment (Fig. 5B–D), more roots were formed when the treatment started with MS medium alone.

The auxin concentration was also important for the second treatment in which the explants were incubated for 48 h with different concentrations of IBA. Again with 1 μM and 10 μM IBA good induction of adventitious rooting was found with up to 95% of the segments showing roots (Fig. 5B). Callus formation without subsequent root formation was observed at concentrations <0.1 μM IBA.

Identification of transcripts expressed during adventitious rooting using differential display

The treatments of *Arabidopsis* stems described above were used to test this system for its suitability to isolate differentially expressed genes during adventitious rooting. Since the experimental procedure allowed the difference between callus formation and adventitious rooting to be distinguished, the comparison of control stems with stems treated to form callus or adventitious roots should provide transcripts which are specific for the rooting process. The

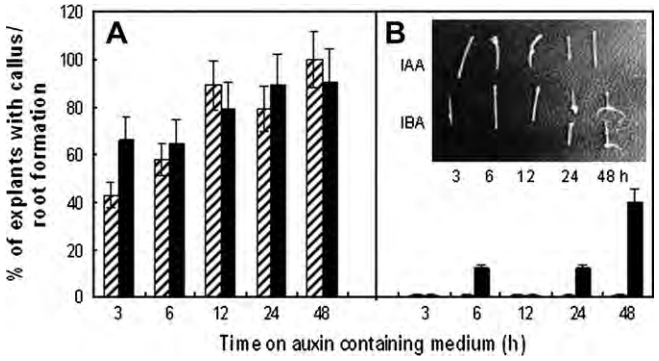

Fig. 3. Callus (A) and root (B) formation after continuous treatment with 10 μM IAA (hatched bars) or IBA (black bars) for different times on MS medium. The photograph shows the phenotype of rooted stem segments incubated for different periods on 10 μM IAA or IBA.

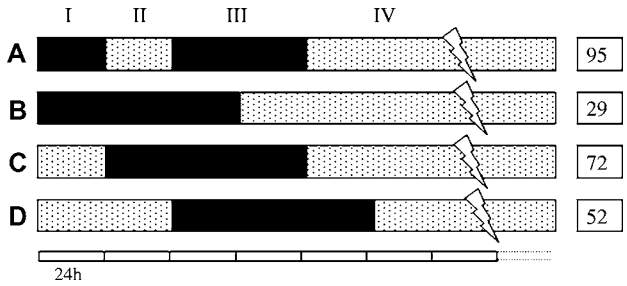

Fig. 4. Adventitious root formation is increased by a two stage treatment and does not require continuous exposure to IBA. Different treatments with IBA are marked as follows: (dotted section) MS only, (black section) MS+10 μM IBA. The different variations tested are: (A) 24 h IBA/24 h MS/48 h IBA/MS; (B) 72 h IBA/MS; (C) 24 h MS/72 h IBA/MS; (D) 48 h MS/72 h IBA/MS. The segments were placed either on MS medium or MS supplemented with IBA after the indicated time periods (see time scale; one white bar segment represents 24 h). Percentage of root formation under the respective treatment conditions is given to the right of the bar. The stages mentioned in the text are indicated above the respective bar in Roman numerals. I: first IBA treatment; II: first period on MS; III: second IBA treatment; IV: remaining time until roots are visible on MS). The flash indicates a discontinuous time scale.

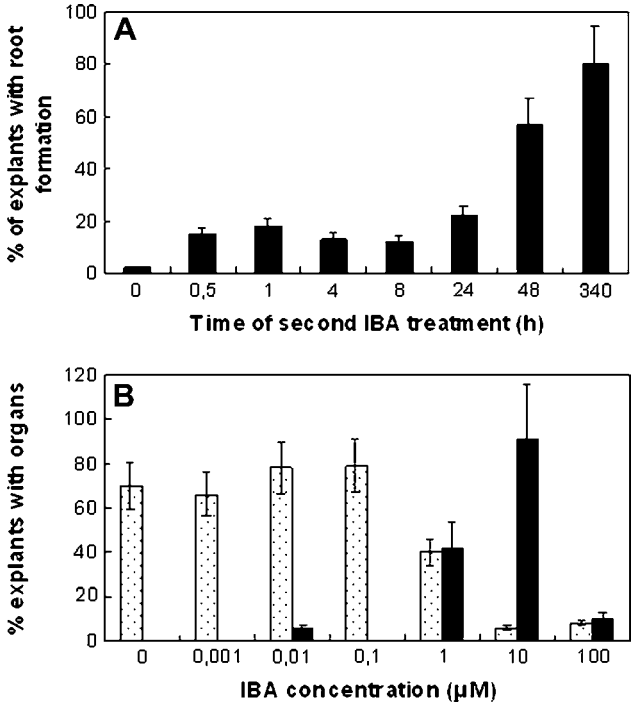

Fig. 5. Two stage treatment for distinction between callus (dotted bars) and root (black bars) formation. (A) Dependence of root formation on the duration of the second 10 μM IBA treatment. Segments were transferred from IBA containing medium to medium without hormone after the respective time periods. (B) Optimum concentration of IBA for the second treatment (340 h). The medium for the second treatment was supplemented with different IBA concentrations.

following three tissue samples were compared: (i) untreated segments; (ii) segments exposed to 10 μM IBA for 24 h, which will induce callus but not root formation; and (iii) segments given 24 h 10 μM IBA, 24 h no IBA, 48 h 10 μM IBA (see Fig. 4, Regime A), which induces roots in a large fraction of the explants (Fig. 6A). For the differential display experiment, a set of arbitrary primers (OPA1-12) was used in combination with anchor primers on each of the three mRNA populations described above. Bands specific to treatment C (root induction) were obtained with OPA primers 1, 6, and 12 (data not shown). Fragments designated 01-a, 01-b, 06-a, and 12-a were excised, reamplified and further analysed. It was not possible to reamplify fragment 12-a, therefore only three differentially expressed fragments remained. In all three cases only short fragments were amplified from the 3′-end. Therefore, all sequences are 3′-UTRs of the respective cDNAs. Since the completion of the *Arabidopsis* genome sequencing project, identification of gene sequences has been much facilitated. One 390 bp fragment (01-a) was homologous to a regulatory subunit B of protein phosphatase 2A (At3g54930). A second 340 bp fragment (01-b) was found to be derived from At1g29470 which was annotated as similar to the early-responsive dehydration stress protein, ERD3 that contains a putative methyltransferase motif. A third 300 bp fragment (06-a) was derived from At5g48545, a gene encoding an unknown protein of the histidine triad family protein with a HIT domain (http://www.tigr.org/tdb/e2k1/ath1/). Expression analysis confirmed the presence of the PP2A homologous mRNA specifically in tissues after IBA-induced adventitious root formation (Fig. 6B).

The polar auxin transport inhibitor TIBA inhibits adventitious root formation

Factors important for the effect of auxins during rooting might be (i) synthesis, (ii) metabolism, and (iii) transport. The latter was tested by using the polar auxin transport inhibitor 3,4,5-triiodobenzoic acid (TIBA) concomitantly with the IBA treatment leading to adventitious roots. Inhibition of root formation was observed when varying

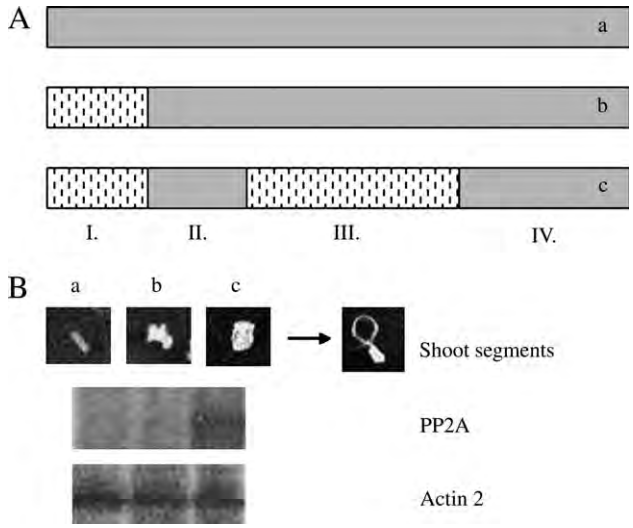

Fig. 6. Identification of transcripts specifically expressed during adven titious root formation by IBA. (A) The different treatment of segments is shown: (a) untreated segments; (b) segments exposed to 10 μM IBA for 24 h, which will induce callus but not root formation; (c) segments given 24 h 10 μM IBA, 24 h no IBA, 48 h 10 μM IBA. (dashed section) Time on IBA, (grey section) MS only. (B) RNA expression analysis of one fragment (PP2A B regulatory subunit) in the three different tissues. The small photographs (a c) show the segments as they looked after the harvest. Root development in treatment (c) was only observed later (indicated by an arrow). The Roman numerals correspond to those in Fig. 4.

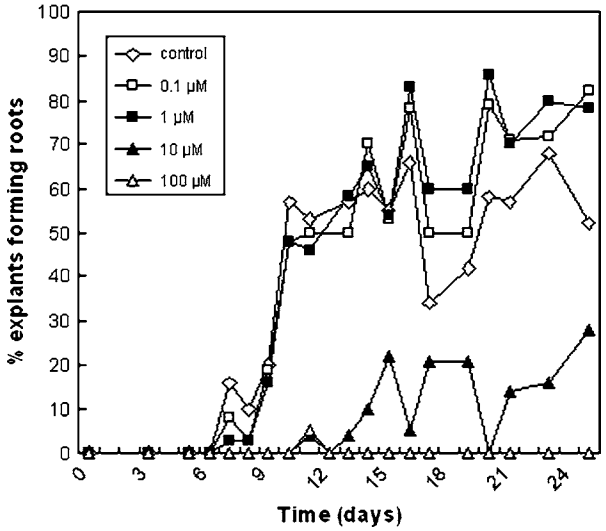

Fig. 7. Adventitious root formation at different concentrations of the IAA transport inhibitor TIBA in wild type plants over a time period of 4 weeks. IBA was always at 10 μM. Inhibition of adventitious root formation was found at equimolar concentrations of IBA and TIBA.

concentrations of TIBA were added together with a fixed concentration of IBA (10 μM) in the medium (Fig. 7). While 0.1 μM and 1 μM TIBA had no inhibitory effect, 10 μM TIBA was already inhibitory and 100 μM TIBA completely prevented adventitious root formation. With lower TIBA concentrations there even seemed to be a small promoting effect after longer incubation times.

Arabidopsis *mutants with altered adventitious root formation*

IBA is an important factor for adventitious root formation if applied exogenously. However, endogenous auxins may also play a role in the rooting process. Therefore three mutants with altered auxin levels were investigated for their ability to form adventitious roots after IBA treatment. The mutant *amt1* (Kreps and Town, 1992) has no altered phenotype compared with the wild type when grown under normal conditions. However, if *amt1* was grown on 10 μM IBA, the roots looked more stunted with a higher number of lateral roots and, at higher concentrations, less root growth than the wild type was observed. *amt1* also showed altered levels of IAA and IBA (Ludwig-Müller *et al*., 1993). It was therefore of interest to test whether this mutant behaved differently concerning adventitious rooting and so at the same time two other mutants with defects in the tryptophan biosynthesis pathway, *trp1* and *trp2* (Last *et al*., 1991; Rose *et al*., 1992) were included. Since adventitious root formation was shown to be concentration-dependent in *Arabidopsis*, several IBA concentrations were tested on wild-type and mutant stem segments. At intermediate IBA concentrations (3–10 μM), root induction was less efficient in *trp1*, a tryptophan auxotroph of *Arabidopsis* with a bushy phenotype but no demonstrable reduction in IAA levels, compared with wild-type Columbia (Fig. 8). The two other mutants (*amt1* and *trp2*) with measurably higher levels of IAA show root induction characteristics very similar to the wild type.

Discussion

Arabidopsis has been used for the investigation of lateral root development (Neuteboom *et al*., 1999) because of its relatively simple organization of both primary and lateral roots (Dolan *et al*., 1993). Lateral root formation in root cultures of *Arabidopsis* was initiated by exogenous auxin. Differential screening of a cDNA library from roots treated with 1-NAA and the inactive analogue 2-NAA led to the isolation of four cDNAs clones coding for proteins putatively active outside the cell such as subtilisin-like serine protease (Neuteboom *et al*., 1993). *Arabidopsis* mutants exhibiting more lateral roots (*sur1*, *sur2*) were linked to an overproduction of IAA (Boerjan *et al*., 1995; Delarue *et al*., 1998). However, other genes regulated independently of auxin induction are also involved in lateral root development, such as the nuclear-localized protein ALF4 (DiDonato *et al*., 2004).

Evidence for the involvement of IBA, but not IAA, in lateral root development was recently reported for lateral root induction in rice (Wang *et al*., 2003). While IBA was

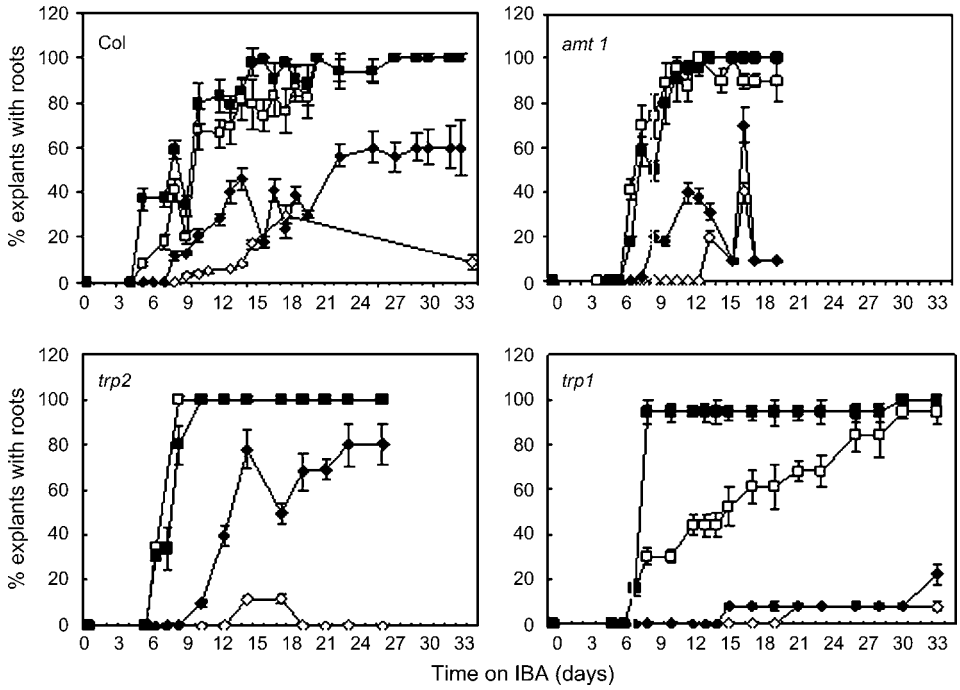

Fig. 8. Adventitious root induction at different IBA concentrations in wild type (Columbia) and three different *Arabidopsis* mutants with altered auxin content over a time period of 5 weeks: (open diamonds) 1 μM, (filled diamonds) 3 μM, (open squares) 10 μM, (filled squares) 30 μM.

able to induce lateral roots, the same response was found only at 20-fold higher concentrations of IAA (Chhun *et al*., 2003, 2004). In addition, a rice lateral rootless mutant *Lrt1* could be rescued by IBA but not IAA treatment (Chhun *et al*., 2003). The mutated gene has yet to be described.

In contrast to lateral root development, adventitious root formation has significant practical implications because of the many plant species that are difficult to root. IBA is now used commercially worldwide to root many plant species (Hartmann *et al*., 1990). However, *Arabidopsis* as a model to study adventitious rooting has so far been neglected. The aim of this study was 2-fold: (i) to analyse the process leading to adventitious roots on *Arabidopsis* stems and to find out which of the two auxins known to be present in *Arabidopsis* are involved in the process and to devise an experimental system which could be used to distinguish between callus and root formation and between IAA and IBA in the rooting process; (ii) to test this system for its use in the isolation of differentially expressed transcripts specifically involved in the rooting process. These transcripts could allow a more detailed analysis of adventitious rooting at the molecular level and help to identify candidate genes important for this process. The possible function of the transcripts isolated in this study for the rooting process will be briefly discussed. Furthermore, this system is also suitable for the analysis of available *Arabidopsis* mutants or chemical inducers or inhibitors of the rooting process.

It was shown that IAA and IBA were able to induce adventitious roots on cuttings of *Arabidopsis* stems if the segments were not removed during the treatment (Fig. 1), whereas removal of the segments from auxin-containing medium to MS medium only resulted in the production of calli with about the same efficiency for both hormones. Callus formation preceded adventitious rooting (Fig. 2). After shorter incubation times only IBA treatment resulted in the formation of roots (Fig. 3), indicating that IBA is an important factor for rooting. Several possibilities exist to explain the better performance of IBA versus IAA (summarized in Epstein and Ludwig-Müller, 1993): (i) higher stability, (ii) differences in metabolism, (iii) differences in transport, and (iv) IBA is a slow release source of IAA.

There is now a great deal of evidence that IBA occurs naturally in plants. The higher stability of IBA, in contrast to IAA, during rooting assays was reported by Nordström *et al*. (1991) which affected both degradation and metabolism. It was therefore suggested that IBA may be a very simple 'conjugate' of IAA and must be converted to IAA by β-oxidation to have an auxin effect. The conversion of IBA to IAA occurs in many plant species, such as *Malus pumila* (Alvarez *et al*., 1989), *Pinus sylvestris* (Dunberg *et al*., 1981), *Populus tremula* (Merckelbach *et al*., 1991), *Pyrus communis* (Baraldi *et al*., 1993), and *Vitis vinifera* and *Olea europaea* (Epstein and Lavee, 1984). However, in microcuttings of *Malus* it was found that IBA was converted to IAA only at very low levels (1%), but IBA itself induced more roots than IAA. This led the authors to suggest that either IBA itself is active or that it modulates the activity of IAA (van der Krieken *et al*., 1992, 1993).

The transport hypothesis is supported by recent findings that IBA and IAA are differently transported in *Arabidopsis* (Rashotte *et al*., 2003). These experiments are in agreement with this study's results using polar auxin transport inhibitors.

Several lines of evidence are now emerging which suggest that part of the effects of IBA are the direct action of the auxin itself (Ludwig-Müller, 2000; Poupart and Waddell, 2000), although other functions may be modulated by the conversion of IBA to IAA via β-oxidation (Zolman *et al*., 2000; Bartel *et al*., 2001). For example, drought and osmotic stress induced the synthesis of IBA and, consequently, the endogenous content of IBA was increased, whereas IAA was less affected (Ludwig-Müller *et al*., 1995). In addition, IBA but not IAA was induced after the inoculation of maize roots with an arbuscular mycorrhizal fungus (Ludwig-Müller *et al*., 1997; Kaldorf and Ludwig-Müller, 2000). In this paper a system was established for the induction of adventitious roots on sterile-grown stem sections of *Arabidopsis thaliana* where IBA induced adventitious roots under conditions where IAA was ineffective (Fig. 3). There was a desire to dissect the rooting process and therefore different time and concentration schemes were used for the optimization of adventitious root formation (Fig. 4), which allowed callus and subsequent root formation to be distinguished (Fig. 5).

The second goal of this research was the identification of differentially expressed transcripts during the rooting process. For this, the differential induction of callus and root on *Arabidopsis* stem segments were used and those treatments were compared with the controls (Fig. 6). Only those transcripts which showed up under treatment C (Fig. 6) were analysed further.

Initial studies on the hydrolytic enzymes found during root formation after IBA treatment in cuttings of mung bean revealed the induction of endo-β-1,4-glucanase (Shoseyov *et al*., 1989), whereas the activities of β-1,3-glucanase and α-amylase were not affected. It was shown by *in situ* hybridization that the genes for endo-β-1,4-glucanase were expressed in the area of adventitious root primordia formation and in the cortex, where maceration of the cell walls was in progress in order to enable root emergence through the hypocotyl. To detect the induction of genes during adventitious root formation in loblolly pine (*Pinus taeda*) after treatment with IBA, a non-targeted approach via differential display reverse transcription-polymerase chain reaction was carried out (Hutchison *et al*., 1999). One of the clones isolated by this method showed strong similarity to the α-expansin gene family of angiosperms and the differential gene expression after IBA treatment was confirmed by RNA blot analysis. Expansins are thought to be responsible for acid-induced cell wall loosening and are expressed in rapidly growing tissues (Cosgrove and Li, 1993; McQueen-Mason, 1995). They were reported to be induced in loblolly pine in non-growing regions of the stem prior to the resumption of cell division leading to the appearance of adventitious roots (Hutchison *et al*., 1999).

One fragment differentially expressed during the adventitious rooting process in *Arabidopsis* (Fig. 6B) was identified as a regulatory subunit B of protein phosphatase 2A. In plants, type 2A serine/threonine protein phosphatases (PP2As) are critical in controlling the phosphorylation state of proteins involved in such diverse processes as metabolism, cell–cell communication, response to hormone, and auxin transport (Smith and Walker, 1996). The specificity, activity and subcellular targeting of PP2A is modulated by its association with the A and B subunits (Kamibayashi *et al*., 1994). In *Arabidopsis*, three families of B-type regulatory subunits were identified, each consisting of more than one member (Corum *et al*., 1996; LaTorre *et al*., 1997; Rundle *et al*., 1995; Sato *et al*., 1997). Expression analysis indicated that, in plants, every B subunit shows a widespread, but fine-tuned, expression pattern in different organs (Thakore *et al*., 1999). The function of PP2A during polar auxin transport has recently received more attention (Muday and DeLong, 2001, and references therein). One *Arabidopsis* mutant that provided insight into the regulation of auxin transport is called *roots curl in NPA1* (*rcn1*). This mutant was isolated using an assay for alterations in differential root elongation in the presence of the auxin transport inhibitor NPA aimed at isolating genes encoding proteins involved in auxin transport or its regulation. The *RCN1* gene encodes a regulatory A subunit of PP2A and the *rcn1* mutant exhibits reduced PP2A activity in extracts (Deruère *et al*., 1999). The phenotypic alterations in this mutant are consistent with reductions in PP2A activity because treatment of wild-type plants with the phosphatase inhibitor cantharidin produces a phenocopy of *rcn1*. The *RCN1* gene is expressed in the seedling root tip, the site of basipetal transport, in lateral root primordia, and in the pericycle and stele, the likely site of acropetal transport (Muday and DeLong, 2001). It can be hypothesized that other PP2A subunits are co-ordinately expressed and that polar auxin transport also plays a role in adventitious root formation in *Arabidopsis*. This assumption is supported by the observation here that the auxin transport inhibitor TIBA inhibited adventitious root formation. Deduced from the findings summarized above a role can be proposed for PP2A in the regulation of auxin transport during adventitious rooting by altering the phosphorylation status of proteins involved in these processes thus most likely acting upstream of auxin transport. Auxin transport itself might be important for adventitious rooting by increasing local auxin concentrations.

A second fragment was identified as derived from an early-responsive dehydration stress ERD3 with otherwise unknown function (http://www.tigr.org/tdb/e2k1/ath1/). The sequence contains also a methyltransferase motif. Protection against dehydration may result in an increase

of lateral or adventitious root formation. It was shown that IBA synthesis was increased under drought stress in maize (Ludwig-Müller *et al*., 1995) and the root system under these conditions was shorter, but with considerably more lateral roots. Drought rhizogenesis is an adaptive strategy that occurs during progressive drought stress and is characterized in *Arabidopsis* and other Brassicaceae and related families by the formation of short tuberized hairless roots (Vartanian *et al*., 1994). These roots are capable of withstanding a prolonged drought period and give rise to a new functional root system upon rehydration. IBA might play a role during this process by inducing new roots. This protein might therefore play a more general role in IBA-induced root formation. As long as the function of ERD3 is unclear, this has to remain a hypothesis.

The Histidine Triad (HIT) motif identified in the third gene product, His-phi-His-phi-His-phi-phi (phi, a hydrophobic amino acid), was identified as being highly conserved in a variety of organisms (Seraphin, 1992). The crystal structure of rabbit Hint (histidine triad nucleotide-binding protein), purified as an adenosine and AMP-binding protein, showed that proteins in the HIT superfamily are conserved as nucleotide-binding proteins (Brenner *et al*., 1997). Hint homologues hydrolyse adenosine 5′ monophosphoramide substrates and function as positive regulators of Cdk7/Kin28 *in vivo* (Bieganowski *et al*., 2002), and Fhit (fragile histidine family) homologues related to the HIT family are diadenosine polyphosphate hydrolases (Barnes *et al*., 1996). Therefore, the role of this protein during adventitious root formation might be in the regulation of the cell cycle or in signal transduction pathways.

In conclusion, it has been shown that it was possible to dissect the adventitious root formation process in *Arabidopsis* in such a way as to distinguish between the action of the two auxins IAA and IBA and to establish conditions where one hormone treatment arrests the process at the callus formation stage, whereas a second hormone treatment induces the formation of roots from these calli. In addition, it has been shown that the experiments presented here are a promising method to identify IBA-induced transcripts during adventitious root formation in the model plant *Arabidopsis thaliana*. To study the process of adventitious root formation further, several experiments can be envisioned: (i) the isolation of additional differentially expressed fragments from this screen, or using the now available microarrays to increase the number of cDNAs; (ii) using this screening method to identify *Arabidopsis* mutants impaired in adventitious root formation; and (iii) using known *Arabidopsis* mutants to investigate their response to IBA in this system. The gene sequences identified can then be used to probe the adventitious rooting pathway in horticulturally important species that are difficult to root.

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Minireview

Indole-3-butyric acid in plants: occurrence, synthesis, metabolism and transport

Ephraim Epstein and Jutta Ludwig-Müller

Epstein, E. and Ludwig-Müller, J. 1993. Indole-3-butyric acid in plants: occurrence, synthesis, metabolism and transport. – Physiol. Plant. 88: 382–389.

Indole-3-butyric acid (IBA) was recently identified by GC/MS analysis as an endogenous constituent of various plants. Plant tissues contained 9 ng g^{-1} fresh weight of free IBA and 37 ng g^{-1} fresh weight of total IBA, compared to 26 ng g^{-1} and 52 ng g^{-1} fresh weight of free and total indole-3-acetic acid (IAA), respectively. IBA level was found to increase during plant development, but never reached the level of IAA.

It is generally assumed that the greater ability of IBA as compared with IAA to promote rooting is due to its relatively higher stability. Indeed, the concentrations of IAA and IBA in autoclaved medium were reduced by 40% and 20%, respectively, compared with filter sterilized controls. In liquid medium, IAA was more sensitive than IBA to non-biological degradation. However, in all plant tissues tested, both auxins were found to be metabolized rapidly and conjugated at the same rate with amino acids or sugar.

Studies of auxin transport showed that IAA was transported faster than IBA. The velocities of some of the auxins tested were 7.5 mm h^{-1} for IAA, 6.7 mm h^{-1} for naphthaleneacetic acid (NAA) and only 3.2 mm h^{-1} for IBA. Like IAA, IBA was transported predominantly in a basipetal direction (polar transport). After application of ^{3}H-IBA to cuttings of various plants, most of the label remained in the bases of the cuttings. Easy-to-root cultivars were found to absorb more of the auxin and transport more of it to the leaves.

It has been postulated that easy-to-root, as opposed to the difficult-to-root cultivars, have the ability to hydrolyze auxin conjugates at the appropriate time to release free auxin which may promote root initiation. This theory is supported by reports on increased levels of free auxin in the bases of cuttings prior to rooting. The auxin conjugate probably acts as a 'slow-release' hormone in the tissues. Easy-to-root cultivars were also able to convert IBA to IAA which accumulated in the cutting bases prior to rooting. IAA conjugates, but not IBA conjugates, were subject to oxidation, and thus deactivation. The efficiency of the two auxins in root induction therefore seems to depend on the stability of their conjugates. The higher rooting promotion of IBA was also ascribed to the fact that its level remained elevated longer than that of IAA, even though IBA was metabolized in the tissue.

IAA was converted to IBA by seedlings of corn and *Arabidopsis*. The K_m value for IBA formation was low (approximately 20 μM), indicating high affinity for the substrate. That means that small amounts of IAA (only a fraction of the total IAA in the plant tissues) can be converted to IBA. It was suggested that IBA is formed by the acetylation of IAA with acetyl-CoA in the carboxyl position via a biosynthetic pathway analogous to the primary steps of fatty acid biosynthesis, where acetyl moieties are transferred to an acceptor molecule. Incubation of the soluble enzyme fraction from *Arabidopsis* with ^{3}H-IBA, IBA and UDP-glucose resulted in a product that was identified tentatively as IBA glucose (IBGlc). IBGlc was detected only during the first 30 min of incubation, showing that it might be converted rapidly to another conjugate.

Key words – Auxin, auxin conjugates, auxin transport, IAA, IAA aspartic acid, IAA glucose, IBA aspartic acid, IBA glucose, rooting.

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Introduction

The auxin indole-3-acetic acid (IAA) was the first plant hormone to be used in rooting (Cooper 1935). In the same year, Zimmerman and Wilcoxon (1935) discovered that several new synthetic auxins, among them indole-3-butyric acid (IBA), also promoted rooting. It was demonstrated that IBA is very effective in promoting rooting of a wide variety of plants, and it is used commercially to root many plant species world-wide (Hartmann et al. 1990). Since its introduction more than 50 years ago, IBA has been the subject of hundreds of experiments and articles. Many of the experiments involved trial and error studies of different concentrations, formulas, additives and treatment durations. Today one can still find varieties and cultivars in almost every species that do not root even after treatment with this auxin, respond to treatment during only part of the growing season, or produce roots only in a fraction of the treated cuttings. In recent years, several attempts were made to understand the role of IBA in the rooting process in plants at the metabolic level. For recent reviews on rooting see Gaspar and Hofinger (1988) and Blakesley et al. (1991). In this minireview we will concentrate on the occurrence, biosynthesis, metabolism and transport of IBA in plants.

Abbreviations – 2,4-D, 2,4-dichlorophenoxyacetic acid; DIAasp, 3-hydroxy-2-indolone-3-acetylaspartic acid; IAA, indole-3-acetic acid; IAGlc, indoleacetyl glucose; IAAsp, indole-3-acetylaspartic acid; IBA, indole-3-butyric acid; IBGlc, indolebutyryl glucose; IBAsp, indole-3-butyrylaspartic acid; IPA, indolepropionic acid; NAA, naphthaleneacetic acid; OxIAAsp, 2-indolone-3-acetylaspartic acid.

Natural occurrence of IBA

Although IBA was identified as a natural product in potato peelings by paper chromatography almost 40 years ago (Blommaert 1954), it is still referred to as a synthetic auxin (Hartmann et al. 1990). During the past 20 years IBA was identified in various plants and tissues (Tab. 1), such as tobacco (Bayer 1969) and *Phaseolus vulgaris* (Brunner 1978); and only recently by GC/MS analysis in pea (Budenoch-Jones et al. 1984, Schneider et al.1985), in cypress (Epstein et al. 1988), and maize (Epstein et al. 1989, Ludwig-Müller and Epstein 1991). An attempt was also made to correlate the infection of plants with growth-influencing bacteria and IBA content (Fallik et al. 1989, Epstein et al. 1991). Very little is known about the actual endogenous concentrations of IBA in plant tissues. Sutter and Cohen (1992) synthesized [^{13}C]indole-[ring 2]-3-butyric acid and used it as an internal standard for the determination of IBA in tobacco leaves by isotope dilution GC/MS. They found that the tissue contained 9 ng g^{-1} fresh weight of free IBA and 37 ng g^{-1} fresh weight of total IBA, compared with 26 ng g^{-1} and 52 ng g^{-1} fresh weight of free and total IAA, respectively. The occurrence of free and conjugated IBA under different growth conditions was studied more thoroughly in plantlets of *Arabidopsis thaliana* grown in liquid medium under sterile conditions (Ludwig-Müller et al. 1993). IBA level increased during the development of the plantlets, but never reached the level of IAA. In seeds of *Arabidopsis*, IBA concentration was higher than that of IAA; both hormones were secreted into the medium. Concentrations of free IBA were between 7 and 25 ng g^{-1} fresh weight, whereas total IBA was in the range of 100 ng g^{-1} fresh weight.

IBA metabolism

It is generally assumed that the greater ability of IBA compared with IAA to promote rooting is due to its relatively higher stability (Hartmann et al. 1990). Robbins et al. (1988) demonstrated that IBA can be stored for 6 months in amber or clear glass at various temperatures (22–25, 6 and 0°C) without significant change in biological activity or breakdown. Nordström et al. (1991) showed that IBA was more stable than IAA in KOH solution in black containers at room temperature. Nissen and Sutter (1990) studied the stability of IAA and IBA under various tissue culture procedures. They showed that the concentrations of IAA and IBA in autoclaved MS (Murashige and Skoog 1962) medium were reduced by 40% and 20%, respectively, compared with filter sterilized controls. Under growth chamber conditions, IAA and IBA losses from both liquid and

Tab. 1. Indole-3-butyric acid as a natural endogenous compound in plants.

Plant material	Detection method	Reference
Potato peelings	Paper chromatography	Blommaert et al. 1954
Tobacco leaves	Gas chromatography	Bayer 1969
Phaseolus hypocotyls	TLC and bioassay	Brunner 1978
Pea root nodules	GC/MS	Budenoch-Jones 1984
Pea root, epicotyl & cotyledons	GC/MS	Schneider et al. 1985
Cypress leaves	GC/MS	Epstein et al. 1988
Maize leaves & kernels	GC/MS	Epstein et al. 1989
Maize roots	GC/MS	Fallik et al. 1989
Maize roots, leaves & coleoptiles	GC/MS	Ludwig-Müller and Epstein 1991
Carrot	GC/MS	Epstein et al. 1991
Arabidopsis thaliana	GC/MS	Ludwig-Müller and Epstein 1992
Tobacco leaves	GC/MS	Sutter and Cohen 1992

agar-solidified MS were significant. In liquid medium, IAA was more sensitive than IBA to non-biological degradation. In the light, IAA and IBA concentrations were reduced by more than 97% and 60%, respectively, compared to a decline by 70% and 30%, respectively, in the dark. In agar-solidified MS, IAA and IBA concentrations were reduced by 45% and 38%, respectively, after 30 days in darkness, and ca 95% and 80%, respectively, after only 3 days in the light. Similar results were obtained after application of auxins to plant tissue. Wiesman et al. (1988) found that labeled IBA and IAA were metabolized rapidly by cuttings of mung bean (*Vigna radiata* L.), and 24 h after application only a small fraction of the radioactivity of both auxins corresponded to the free auxin. Similarly, Pythoud and Buchala (1989) found that only 17% of the ^{14}C-IBA which was applied to the bases of cuttings of trembling aspen (*Populus tremula* L.) was still present as unmodified IBA after 24 h. IAA was metabolized more quickly than IBA by green cuttings (leaves and apex excised) of *P. tremula* L. (Merckelbach et al. 1991). Baraldi et al. (1993) also observed a rapid disappearance of IBA in pear (*Pyrus communis* L.) plantlets propagated in vitro. Only a small fraction of the total extractable radioactivity could be identified as free IBA after 12 h of incubation. In apple shoots cultured in vitro only 5% of IBA and 1% of IAA were found in the free form (van der Krieken et al. 1992a,b).

IBA conjugates

It has been established that natural plant hormones such as IAA, gibberellin, cytokinin and ABA are present in plant material both as free acid and in the form of conjugates (Cohen and Bandurski 1982). All plants that have been studied had most of their IAA in conjugated form, and conjugates may account for over 90% of the IAA in some tissues. Evidence has emerged over the last several years which indicates that conjugates play an important role in auxin physiology and metabolism. They are thought to be involved in transport of the hormone, the storage and subsequent usage of the hormone, protection from enzymatic destruction, and the homeostatic control of the concentration of the hormone within the plant (Bandurski 1980, Cohen and Bandurski 1982). Andrea and Good (1955, 1957) reported that pea sections converted IBA to compounds which had chromatographic properties similar to those of IBA-aspartic acid (IBAsp), IAA-aspartic acid (IAAsp) and IAA. They concluded that IBA was converted by β-oxidation to IAA and that both IBA and IAA (formed from the applied IBA) were conjugated to aspartic acid by pea sections.

Wiesman et al. (1988, 1989) studied the metabolism of IAA and IBA by mung bean cuttings. The cuttings were incubated with ^{14}C-IAA and ^{3}H-IBA and samples were extracted after various periods of incubation and the radioactive compounds were identified. The major metabolite of IAA was identified as IAAsp, whereas IBA was converted to IBAsp and at least two high molecular weight conjugates (probably conjugates with peptides). They proposed that the better rooting ability of IBA was due to the formation of IBAsp which promoted rooting better than IAA (Wiesman et al. 1989) or IAAsp (Plüss et al. 1989). Epstein and Sagee (1992) studied the metabolism of IBA in leaf midribs of citrus (*Citrus reticulata* Blanca). IBA was converted by the midribs to a metabolite that was identified tentatively as an ester conjugate with glucose (IBGlc). Pythoud and Buchala (1989) could not detect any oxidation products of IBA after feeding ^{14}C-IBA to cuttings of *P. tremula*, but noticed the conversion of IBA to a glycosyl conjugate and another conjugate, probably IBA peptide. IAA, on the other hand, was both oxidized by *P. tremula* cuttings and conjugated to aspartic acid to form 2-indolone-3-acetylaspartic acid (OxIAAsp). Tsurumi and Wada (1988) found that seedlings of *Vicia faba* oxidized IAA and conjugated it with aspartic acid to form 3-hydroxy-2-indolone-3-acetylaspartic acid (DIAasp). Plüss et al. (1989) and Merckelbach et al. (1991) identified OxIAAsp in greenwood cuttings of *P. tremula*. Neither product was biologically active (Tsurumi and Wada 1988, Plüss et al. 1989). Riov and Bangerth (1992) identified OxIAAsp in tomato fruit tissue. These reactions render IAA inactive and thereby remove it from the auxin pool. Since no such oxidation products have yet been found for IBA, this might be the cause for the better activity of IBA in rooting. However, it is also possible that IBA oxidation products have yet to be discovered.

Merckelbach et al. (1991) also showed that the IBA was conjugated mainly to IBGlc and that IAA was conjugated more slowly than IBA in cuttings of *P. tremula*, probably because IAA, and not IBA, induced its own metabolism. They ascribed the higher rootability of IBA to the fact that its level remained elevated longer than that of IAA, even though IBA was metabolized in the tissue. They also showed that following incubation with IBA, the level of IAA and IAAsp in the cuttings increased steadily.

Plantlets of easy- and difficult-to-root cultivars of pear grown in vitro converted approximately 50% of the IBA taken in to IBAsp (Baraldi et al. 1993). Baraldi et al. attributed the differences in the rooting ability of the two pear cultivars to differences in IBA uptake and metabolism and to free IAA level in the shoots. Epstein et al. (1993b) studied the metabolism of exogenously-applied IBA in easy- and difficult-to-root cultivars of sweet cherry (*Prunus avium* L.) in an aseptic system. Both cultivars rapidly metabolized the IBA to a conjugate, probably IBGlc. Autofluorography of the extracts of the plantlet bases showed that after one day no free IBA could be detected in the difficult-to-root cultivar, while the easy-to-root cultivar metabolized the IBA more slowly and free IBA could still be detected. The free IBA disappeared after 2 days of incubation, but

re-appeared after 4 days (3 days before root emergence) and disappeared again after 7 days. They postulated that the easy-to-root, as opposed to the difficult-to-root cultivar, has the ability to hydrolyze the ester conjugate at the appropriate time to release free IBA, which may promote root initiation. This theory is supported by reports on increased levels of free auxin in the bases of cuttings prior to rooting (Brunner 1978, Moncousin et al. 1989, Liu and Reid 1992, Epstein and Ackerman 1993. Epstein et al. (1993b) used the conjugate inhibitor 2,6-dihydroxyacetophenone (DHAP, Lee and Starratt 1986) in order to increase the level of free auxin in cuttings of the difficult-to-root olive cv. Uovo di Piccone. Significantly more cuttings rooted following treatment with 2 m*M* DHAP and 0.8% IBA than with 0.8% IBA alone (30% vs 15%, respectively).

IBA was metabolized very rapidly by *Petunia hybrida* cell suspension (Epstein 1993a). HPLC of the cell extracts demonstrated a new metabolite after only 2 min of incubation, and after 30 min 60% of the radioactivity was in the new metabolite vs 10% in the IBA. The new compound was resolved by autofluorography to two metabolites but after 24 h only one metabolite was present. One metabolite was eluted in the neutral fraction from DEAE-Sephadex column and was hydrolyzed to free IBA by both 1 *M* NaOH and β-glucosidase, proving it to be an ester conjugate of glucose with IBA (Bandurski and Schulze 1977). The second metabolite was eluted from the DEAE-Sephadex column with the acidic fraction, hydrolyzed to free IBA by 7 *M* NaOH, but not by 1 *M* NaOH or β-glucosidase, pointing to a peptide bond (Bandurski and Schulze 1977). In a separate experiment, it co-chromatographed with authentic IBA-aspartic acid on TLC. Autofluorography showed that IBGlc was the first to appear without a distinct lag phase simultaneously with the decrease of IBA. After 30 min, IBAsp began to show, and IBGlc decreased and disappeared after 24 h. In *A. thaliana* seedlings cultured in liquid medium, the formation of IAA and IBA conjugates was also inducible by exogenous NAA, but the amount of conjugates formed was dependent on the time of the addition of NAA to the culture medium (Ludwig-Müller and Epstein 1993).

Epstein and Sagee (1992) showed that *Citrus* leaf midribs metabolized exogenous IBA to a compound that was tentatively identified as an ester conjugate. Zenk (1964) claimed, without supporting data, that IAGlc is formed in plants without a lag phase, and that this conjugate is the precursor of amino acid conjugates. Michalczuk and Bandurski (1982) showed that the actual substrate for the IAA-*myo*-inositol forming enzyme was IAGlc. IAA glucose is formed from UDP-glucose by a transferase, and the equilibrium of this reaction is strongly in favor of UDP-glucose by a factor of about 50:1 (R. S. Bandurski, personal communication). IAGlc is thus a highly effective acyl donor and a likely candidate for the acylation of aspartate. In the same fashion, *Petunia* cells rapidly conjugate IBA to IBGlc, which in turn is converted to form IBAasp. It was postulated that IBAasp probably acts as a 'slow-release' hormone (Cholodny 1935). *Petunia* probably releases IBAsp into the medium, and since the cells do not absorb IBAasp readily (Shea et al. 1988), the IBA in the medium is replaced in this way by IBAsp until equilibrium is reached. Only intact cells were able to metabolize IBA and the reaction was affected by low temperature and anaerobic conditions. IAA metabolism proceeded at a slower rate, and autofluorography showed that while free IBA disappeared after 0.5 h, free IAA was still present after 1 h incubation.

Nordström et al. (1991) studied the metabolism of exogenous IAA and IBA during adventitious root formation in pea cuttings. The cuttings metabolized IAA predominantly to IAAsp. The level of free IAA in the cuttings increased considerably on the first day of incubation, but then decreased rapidly and reached control level after 4 days. IBA, on the other hand, was converted to IAA, IBAsp and IAAsp. Free IBA level remained high throughout the experimental period.

In seedlings of *A. thaliana* cultivated in liquid medium under sterile conditions, IBA was also metabolized to two major metabolites (Ludwig-Müller and Epstein 1993) that were tentatively identified as IBGlc and IBAsp. Incubation of the soluble enzyme fraction from *A. thaliana* with ^{3}H-IBA, IBA and UDP-glucose resulted in a product with an R_f value identical to that of IBGlc and which stained positive for indole. IBGlc was detected only during the first 30 min of incubation, showing that it might be rapidly converted to another conjugate, as was also demonstrated with IAA in maize (Kowalczyk and Bandurski 1991). This was the first report of an enzyme that is able to conjugate IBA in vitro.

Conversion of IBA to IAA

Fawcett et al. (1960) found that indolealkenecarboxylic acids with even chain lengths were converted in the plant to acetate, and those with odd chain lengths resulted primarily in propionate. They exposed wheat coleoptile and pea stem tissue to IBA solutions and subsequently identified IAA in the tissues and the residual solution. Conversion of IBA to IAA was reported in cuttings of *Pinus sylvestris* (Dunberg et al. 1981), *P. tremula* (Merckelbach et al. 1991), *P. communis* (Baraldi et al. 1993), *Malus pumila* (Alvarez et al. 1989) and *Malus* cultivar Jork (van der Krieken et al. 1992a). Epstein and Lavee (1984) used ^{3}H-IBA to demonstrate the conversion of IBA to IAA in cuttings of grapevine and olive. Noiton et al. (1992) found that the level of IAA increased markedly in apple microcuttings after treatment with IBA. Although the conversion of IBA to IAA was found to occur in so many plant species, and many authors attribute the rooting ability of IBA to its conversion to IAA, no biochemical studies

of this reaction have, to the best of our knowledge, been carried out.

Biosynthesis of IBA

The biosynthesis of IBA can proceed according to one of the following pathways: (1) a pathway analogous to the IAA biosynthetic pathway via the tryptophan pathway (indole + serine) using glutamate-γ-semialdehyde instead of serine; (2) by β-oxidation via reactions similar to those found in the biosynthesis of fatty acids; (3) a nontryptophan pathway similar to that demonstrated by Wright et al. (1991) in a mutant of the maize orange pericarp for IAA.

IAA was found to be converted to IBA by seedlings of corn (*Zea mays* L.) (Ludwig-Müller and Epstein 1991). After feeding [1-^{14}C]-IAA to segments of corn shoots and roots, a new labeled compound was detected by TLC and HPLC which corresponded to authentic IBA. This compound was not detected in a control experiment without plant tissue. After feeding [$^{13}C_6$]-IAA to the plant tissues it was possible to identify the formation of [$^{13}C_6$]-IBA by GC/MS. Most of the conversion was found to occur in the leaves and some activity was found also in the roots and coleoptiles. Maximum activity was detected after 1 h incubation, and the pH optimum was 6.0 for uptake and 7.0 for IBA formation. The K_m value for IBA formation was low (approximately 20 μM), indicating high affinity for the substrate. That means that small amounts of IAA (only a fraction of the total IAA in the plant tissues) can be converted to IBA. It was also shown that the conversion rate was higher in a variety of corn that formed an extensive root system, than in another variety with smaller roots. It is suggested that IBA is formed by the acetylation of IAA with acetyl-CoA in the carboxyl position via a biosynthetic pathway analogous to the primary steps of fatty acid biosynthesis, where acetyl moieties are transferred to an acceptor molecule (Wakil 1989).

Several co-enzymes which might be involved in the chain elongation reaction were studied as to their effects on IBA formation in vitro (Ludwig-Müller and Epstein 1992, 1993). In the presence of the co-factors ATP, Mg^{2+}, and NAD(P)H, some IBA forming activity was detected with acetyl-CoA and propionyl-CoA, but not with malonyl-CoA or acetoacetyl-CoA. A labeled product was detected only when the 50000 g fraction was incubated with acetyl-CoA or propionyl-CoA and its formation was increased by adding ATP or Mg^{2+}, respectively. A time course study of the formation of this product showed that it must be turning over, but it is not directly converted to IBA or IPA in this fraction. Preliminary characterization of the product indicated that it is an indole, most likely a carboxylic acid, and it is probably a conjugate. The product was collected by HPLC and incubated with the 10000 g fraction and the supernatant. IBA-forming activity was detected in the 10000 *g* fraction and found to be enhanced by NADPH. The supernatant also showed significant activity without any co-factor, but the product was unstable. In the organelle fraction, no IBA formation was detected after 1 h incubation, but after 4 h approximately 45% of the product was converted to IBA. It was therefore concluded that the biosynthesis of IBA might be a two-step reaction, but further characterization and identification of the intermediate step is necessary to confirm this hypothesis.

Incubation of axenically cultured seedlings of *Arabidopsis* with labeled IAA resulted in a labeled compound with an R_f similar to authentic IBA (Ludwig-Müller and Epstein 1993). The IBA peak decreased rapidly concurrently with the formation of a new compound which was identified as an IBA conjugate. Determination of the acidic organic phase showed that after 1 h incubation with IAA most of the IAA that was taken in (46%) was found as an IAA conjugate, a significant amount (ca 24%) was in IBA conjugates, and only ca 14% of the recovered radioactivity was present as free IBA. In contrast, van der Krieken et al. (1992a) did not find any conversion of IAA to IBA by shootlets of apple grown in vitro.

Uptake and transport of IBA

Studies of the transport of IBA using the *Avena* curvature test showed that IAA was transported faster than IBA (Went and White 1938, McCready 1963). Among the various auxins, IAA and NAA appeared to have very similar transport velocities (Hertel et al. 1969, Kaldewey 1984), while IBA was markedly slower (McCready 1963), behaving more like 2,4-D (Riov and Goren 1979). Leopold and Lam (1961) determined the rate of the polar movement of some auxins, and found that the velocity of NAA was 6.7 mm h^{-1}, of IAA 7.5 mm h^{-1} and of IBA only 3.2 mm h^{-1}.

IBA was transported in midribs of *Citrus* leaves predominantly in a basipetal direction (polar transport), at a somewhat lower rate than IAA (Epstein and Sagee 1992). Basipetal transport of IBA was approximately 60% of that of IAA and only twice the acropetal transport. After application of ^{3}H-IBA to cuttings of *V. radiata* (Wiesman et al. 1988), *P. tremula* (Pythoud and Buchala 1989), olive and grapevine (Epstein and Lavee 1984), sweet cherry (Epstein et al. 1993b), and apple (van der Krieken et al. 1992b), most of the label remained in the bases of the cuttings. The same results were obtained using labeled IAA (Pythoud and Buchala 1989) and [$^{13}C_6$]-IAA (Liu and Reid 1992). This suggests that the auxin transport is passive and is probably involved with xylem transport, which is probably due to transpiration. Pythoud and Buchala (1989) showed that IBA was associated with the cambium and phloem tissues. It was suggested (Jarvis and Booth 1981) that basal application of IBA to mung bean cuttings increased the basipetal translocation of IAA that was

applied to the leaves. Pythoud and Buchala (1989) used the agar block technique with ^{14}C-IAA in the donor block at the apical end, and found no significant increase in the translocation of IAA in stem segments or petioles of *P. tremula* when 10 mg ml^{-1} of IBA was present in the receiver block. However, when they used stem segments from cuttings which were pretreated with 10 mg ml^{-1} of IBA for 24 h they noticed an increase in the translocation of IAA. In an experiment with *L. discolor* (Epstein and Ackerman 1993), cuttings of easy- and difficult-to-root cultivars were incubated with labeled IBA. The major difference in the transport between the two cultivars was that in the difficult-to-root cultivar the label in the leaves was almost a constant 10% of the total uptake, while in the easy-to-root leaves the value increased after 2 weeks to 30% and after 3 weeks to 45% , with a final level of 35% after 4 weeks (the same as in the shoot).

In a study of IBA uptake by easy- and difficult-to-root plantlets of sweet cherry (Epstein et al. 1993b) it was found that easy-to-root plantlets absorbed more IBA for a longer period than did difficult-to-root plantlets. The uptake of IAA and IBA was studied in suspension cell cultures of *P. hybrida* (Epstein et al. 1993a). The initial uptake of ^{3}H-IBA was much higher than that of ^{3}H-IAA, and after 10 min of incubation with labeled IBA and IAA , 4.6 p*M* vs 0.35, respectively (39% vs 12% of total applied radioactivity) was found in the cell extracts. The uptake of IBA reached a plateau of 6.0 p*M* (62%) after 2 h while that of IAA increased continuously up to 1.5 p*M* (46%) after 24 h. Following the addition of 40 μ*M* of unlabeled auxin more IBA was taken in initially than IAA (39% vs 12%), but the level almost equalized after 24 h incubation when IBA uptake reached 890 n*M* (55%) and IAA 840 n*M* (46%).

Uptake of IAA and IBA in *A. thaliana* was followed for 24 h and 48 h, respectively (Ludwig-Müller and Epstein 1993). There was no significant difference in the uptake of the auxins during these time periods.

The physiological events leading to root initiation may be revealed by studies of the direct genetic controls using molecular genetic techniques. Shoseyov et al. (1989) treated 20-day-old cuttings of mung bean (*V. radiata*) with IBA and checked the activity of hydrolytic enzymes. They found that the activity of endo-1,4-β-glucanase was enhanced only in the IBA-treated cuttings, reaching a maximum 12 h after the IBA treatment. The activities of β-1,3-glucanase and α-amylase were not affected by the treatment. Four endo-1,4-β-glucanase genes were cloned and sequenced. In situ hybridization revealed the expression of the 4 genes in the area of adventitious root primordia formation and in the cortex, where maceration of the cells was in process, to enable emergence of roots through the hypocotyls.

Conclusions

This review has dealt mainly with recent work on IBA in plants. There is a plethora of evidence available showing that IBA is present as an endogenous constituent in a variety of plants and tissues. Still, there is not enough information on its importance as an auxin. In all studies IBA was conjugated very rapidly, and so far no non-conjugation products of IBA have been reported. It is reasonable to assume that free auxin released from the conjugate is the major source of the free auxin

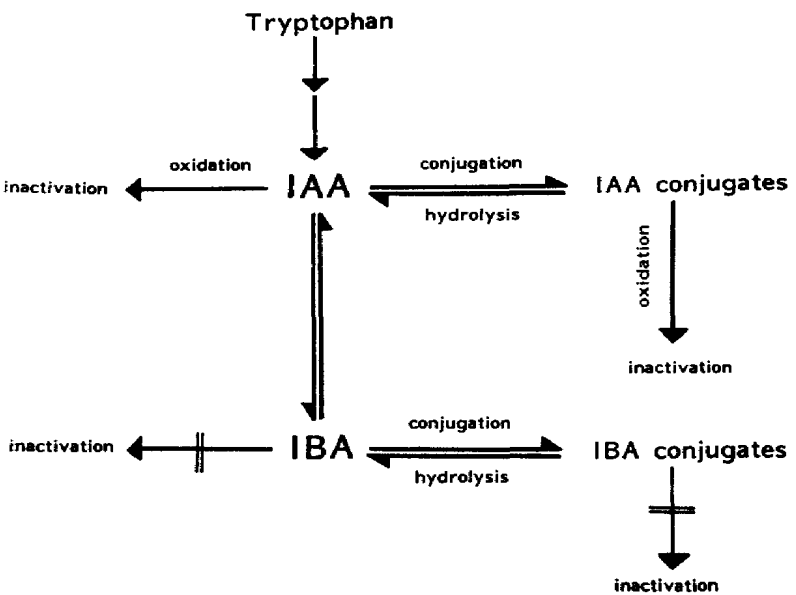

Fig. 1. Scheme for the metabolic interconversions of IAA and IBA and their conjugates.

during the rooting process. The interconversions of IAA and IBA (Fig. 1), the different conjugates in various plants, and the influence of factors such as medium, light, pH, and others on its metabolism makes it difficult to construct a hypothesis for the involvement of IBA in adventitious root initiation. There are several possibile explanations for the better rooting ability of IBA than of IAA, and the failure of some plants to root even after IBA treatment: (1) IBA shows better stability in solution; (2) rooting ability depends on the formation of IAA from IBA and a certain ratio of IAA:IBA must be maintained; (3) rooting ability depends on the stability of the respective auxin conjugates; (4) rooting ability depends on the ability of the tissue to convert the auxin conjugate to the free hormone during a critical rooting phase; and (5) uptake and transport of the two auxins differ significantly. There is still not enough evidence to support any of the hypotheses, and more work is needed to elucidate the role of IBA as an auxin and rooting factor in plants.

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Characterization and partial purification of indole-3-butyric acid synthetase from maize (*Zea mays*)

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In previous work it has been shown that the route from indoleacetic acid (IAA) to indolebutyric acid (IBA) is likely to be a two-step process with an unknown intermediate designated 'product X'. Our objective was to characterize and purify enzyme activities that are involved in these reactions. Indole-3-butyric acid synthetase was isolated and characterized from light-grown maize seedlings (*Zea mays* L.), which were able to synthesize IBA from indole-3-acetic acid (IAA) with ATP and acetyl-CoA as cofactors. The enzyme activity is most likely located on the membranes of the endoplasmic reticulum, as shown by means of aqueous two-phase partitioning and sucrose density gradient centrifugation, with subsequent marker enzyme analysis. It was possible to solubilize the enzyme from the membranes with a detergent (CHAPS) and high concentrations of NaCl. The molecular mass of solubilized IBA synthetase was ca 31 kDa and its isoelectric point was at pH 4.8. The enzyme forming the reaction intermediate had a molecular mass of only 20 kDa and it seemed to be located on different membranes. Inhibition experiments with reducing agents and sulfhydryl reagents indicated that no sulfhydryl groups or disulfide bridges were present in the active centre of IBA synthetase. KCN inhibited the enzyme activity completely, and sodium azide by about 50%. Substrate analogs, such as 1-IAA, 2,4-dichlorophenoxyacetic acid, phenylacetic acid, and naphthaleneacetic acid, inhibited IBA formation to a high extent. Experiments with tunicamycin gave evidence that the enzyme is not a glycoprotein. These findings were confirmed by affinity chromatography with Concanavalin A, where the enzyme did not bind to the matrix. Further purification of the IBA synthetase on an ATP-affinity column resulted in a more than 1 000-fold purification compared to the microsomal membranes. IBA synthetase activity was also present in other plant families. Our results present further evidence that IBA is synthesized by a two-step mechanism involving two different enzyme activities.

Key words – Acetylcoenzyme A, fatty acid biosynthesis, indole-3-acetic acid, indole-3-butyric acid biosynthesis, light, *Zea mays*.

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Introduction

Although indole-3-butyric acid (IBA) is a very prominent auxin in horticultural practice, it was only recently established that it is a native compound in various species (Epstein et al. 1989, Ludwig-Müller and Epstein 1991, Sutter and Cohen 1992, Ludwig-Müller et al. 1993).

The biosynthesis of IBA might proceed via one of the following pathways: (1) a pathway analogous to indole-3-acetic acid (IAA) via a tryptophan analog with longer side chain, (2) by chain elongation reactions similar to those found in fatty acid biosynthesis (Wakil 1989) using IAA as substrate, or (3) by a non-tryptophan pathway similar to that demonstrated by Wright et al. (1991) for IAA in a mutant of maize. The question of the possible precursor for IBA in plants was first addressed in maize. Ludwig-Müller and Epstein (1991) demonstrated for the first time that IAA is converted to IBA in vivo using dark-grown maize seedlings. Using differently labelled IAA as substrate, it was possible to identify the

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reaction product IBA by three independent methods (TLC, HPLC, and GC-MS). Most of the conversion of IAA to IBA was found to occur in the leaves, compared to coleoptile and root segments. The reaction was recently demonstrated to occur in *Arabidopsis thaliana* seedlings and mature plants (Ludwig-Müller and Epstein 1994) and two varieties of sterile cultured *Grevillea* (J. Ludwig-Müller, unpublished results), but it was not found in apple cuttings and stem slices (van der Krieken et al. 1992, 1993). It was suggested that IBA is formed by the acetylation of IAA with acetyl-CoA in the carboxyl group, because in labelling experiments the carboxy-^{14}C was retained (Ludwig-Müller and Epstein 1991). Investigations on the in vitro conversion of IAA to IBA in maize seedlings revealed that the biosynthesis of IBA proceeds via chain elongation reactions (pathway 2) and that the in vitro reaction is dependent on a microsomal membrane preparation. In light-grown maize seedlings IBA was formed from IAA with acetyl-CoA and ATP as cofactors, whereas in dark-grown seedlings under the same reaction conditions an intermediate reaction product with indole characteristics was formed (Ludwig-Müller and Epstein 1992, Ludwig-Müller et al. 1995a). This compound was converted to a reaction product with the retention time of IBA by an organelle (10 000 *g*) fraction from dark-grown maize seedlings (Ludwig-Müller and Epstein 1992, Ludwig-Müller et al. 1995a). Therefore, we have proposed that the biosynthesis of IBA might be a two step process with a yet unidentified intermediate referred to as 'product X'. First attempts to identify this unknown reaction product have indicated the possibility of a conjugate of IAA with ADP (Ludwig-Müller et al. 1995a). Recently, it has been shown that IBA biosynthesis might be regulated by drought stress and ABA (Ludwig-Müller et al. 1995b) in maize seedlings. In this study the enzyme(s) involved in the conversion of IAA to product X and of IAA to IBA are further characterized and an IBA synthetase is partially purified.

Abbreviations – ABP, auxin binding protein; ACP, acyl carrier protein; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; IBA, indole-3-butyric acid; NAA, naphthylacetic acid; NEM, N-ethylmaleimide; 3-OMG, 3-O-methyl-D-glucopyranoside; PAA, phenylacetic acid; PCMBS, *p*-chloromercuriphenylsulfonate.

Materials and methods

Plant material

Seedlings of maize (*Zea mays* L. cv. Ascot), pea (*Pisum sativum* L. cv. Schnabel), cucumber (*Cucumis sativus* L. cv. Delikatess), sunflower (*Helianthus annuus* L. cv. Hohes Sonnengold), Chinese cabbage (*Brassica campestris* ssp. *pekinensis* cv. Granat), wheat (*Triticum aestivum* L. cv. Ibis), tomato (*Lycopersicon esculentum* L. cv. Hilds Frühstamm), and carrot (*Daucus carota* L. cv. Nantaise) were cultivated under sterile conditions (Ludwig-Müller and Epstein 1991), both in the dark and under continuous illumination (28 μmol m^{-2} s^{-1}; Philips TL55 and TL32) at 23°C. *Arabidopsis thaliana* L. (Heynh.) Columbia ecotype and tobacco (*Nicotiana tabacum* L. cv. Atropurpurea) seedlings were grown under sterile conditions in liquid shaking culture (Ludwig-Müller et al. 1993). The plant material was routinely harvested after 6 days of culture.

Preparation of the microsomal fraction

The isolation of microsomal membranes was carried out by a method as modified by Ludwig-Müller et al. (1995b), using 50 m*M* HEPES buffer, pH 7.0, containing 0.1% (w/v) bovine serum albumin (BSA) and 250 m*M* sucrose as homogenisation medium. Only shoot material of light- and dark-grown maize was used in this study, if not otherwise indicated. The 50 000 *g* pellet was resuspended in 50 m*M* HEPES, pH 7.0, containing 250 m*M* sucrose. All operations were carried out at 4°C.

Membrane purification

The resuspended membranes were either directly used for the enzyme assay or for further identification separated on an aqueous two-phase system or on a sucrose step gradient. The aqueous two-phase system (Albertsson et al. 1982) consisted of Dextran 500 and polyethyleneglycol in a buffer containing 5 m*M* K_2HPO_4 and 250 m*M* sucrose, pH 7.7. Polymer concentrations between 6.0 and 8.5% (w/w) of each polymer were used to determine the optimum concentration for the separation of the maize membranes. The procedure for the individual concentrations was carried out as described by Ludwig-Müller and Hilgenberg (1988). Sucrose gradient centrifugation was performed on a step gradient of 18, 26, 31, 36, and 45% (w/w) sucrose. In some experiments a gradient of 31, 36, 45, 50, and 55% sucrose, or a combination of both gradients was used. Centrifugation was for 60 min at 100 000 *g*. The individual bands were collected, diluted with HEPES-sucrose buffer, pH 7.0 (1:10) and the membranes of each fraction pelleted for 60 min at 50 000 *g*. The pellet was then resuspended in the same buffer.

For further experiments the IBA synthetase containing membrane pellets were solubilized either with 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, (CHAPS; 15 m*M*) or with 1 *M* NaCl for 30 min at 4°C. Membranes containing product X-forming enzyme activity were solubilized with 1% (w/v) Tween 20 under the same conditions. The extracts were then centrifuged for 30 min at 50 000 *g*, the pellet discarded and the supernatant collected.

Gel filtration chromatography

Solubilized proteins were analyzed on a silica gel column with bound diol phase (GF 250, Zorbax, Du Pont, Bischoff Analysentechnik GmbH, Leonberg, Germany) and 100 m*M* Na_2HPO_4, pH 7.5, as solvent. Flow rate was 0.8

ml min^{-1} and detection of proteins was by absorbance at 280 nm. The system was calibrated with the following proteins: γ-globulin (160 kDa), γ-globulin subunit (80 kDa) BSA (66 kDa) egg albumin (45 kDa) and chymotrypsinogen (25 kDa). Fractions of 0.4 ml were collected and assayed for synthesis of IBA and product X.

Affinity chromatography

ATP-agarose column

The active fraction after gel filtration was applied on an ATP affinity column (Sigma-Aldrich, Deisenhofen, Germany; 1 ml column volume), pre-equilibrated with 50 m*M* HEPES, pH 7.0, containing 5 m*M* $MgSO_4$. The column was washed with excess buffer and the proteins bound to the column were eluted with 2 ml 50 m*M* ATP.

PAA- and IAA-Sepharose column

The columns were prepared using epoxy activated Sepharose 6B (Sigma-Aldrich) and 4-hydroxy-PAA or 5-hydroxy-IAA (D. Klämbt and G. Viola, personal communication). The column was equilibrated with 10 m*M* sodium citrate and 5 m*M* $MgSO_4$, pH 6.0. The bound proteins were eluted with either 1 m*M* naphtylacetic acid (NAA), phenylacetic acid (PAA) or IAA.

Concanavalin A-Sepharose column

Concanavalin A-Sepharose (Sigma-Aldrich) was equilibrated with 100 m*M* sodium acetate, pH 6.0, containing 1 *M* NaCl and 1 m*M* $MnCl_2$. The proteins were eluted with a discontinuous gradient of 3-O-methyl-D-glucopyranoside (3-OMG) between 5 and 100 m*M*.

All protein extracts collected from the affinity columns were either desalted on Sephadex G-25 or dialyzed against H_2O overnight at 4°C.

Isoelectric focusing

A semi-preparative isoelectric focusing (IEF) was carried out according to Righetti (1987) with some modifications, using Sephadex G-75 superfine (Pharmacia, Freiburg, Germany) as matrix. Two g of Sephadex G-75 were mixed with 25 ml distilled H_2O containing the ampholytes (1.3 ml Servalyt 4-9T and 0.5 ml Servalyt 3-10) and the solubilized protein sample (max. 5 mg protein per gel). The mixture was homogeneously distributed under soft shaking in a container (15 × 5 cm × 3–4 mm). The anode solution consisted of 25 m*M* aspartic acid, 25 m*M* glutamic acid, and the cathode solution of 25 m*M* arginine, 4 m*M* lysine, and 2 *M* ethylenediamine. The IEF was performed on precooled plates (4–6°C) and the temperature was maintained during the run. The focusing program was started at 200 V increasing to constant voltage during a run time of 3–4 h. The gel was then divided into 1-cm strips, the Sephadex G-75 resuspended in 2 ml distilled H_2O and the supernatant quantitatively removed. In the supernatant the pH value as well as the enzyme activities were determined.

Polyacrylamide gel electrophoresis

For gel electrophoresis all samples were dialyzed against H_2O overnight at 4°C and, if necessary, concentrated by lyophilisation. Native gel electrophoresis was carried out according to Maurer (1968) on a 12.5% gel. Protein staining was performed with silver nitrate according to Heukeshoven and Dernick (1985).

Marker enzyme analysis

All fractions from the sucrose density gradient were assayed for nitrate-insensitive and K^+-stimulated ATPase at pH 6.5 (Jochem and Lüttge 1987), PP_i-ase (Marquardt and Lüttge 1987), UDPase (Nagahashi and Kane 1982), antimycin A-insensitive NADH-cytochrome *c* reductase (Briskin et al. 1987) and cytochrome *c* oxidase (Briskin et al. 1987). Phosphate release was determined according to Jochem and Lüttge (1987).

Enzyme assay for the formation of IBA and product X

The enzyme assay for the conversion of IAA to product X and IBA was performed according to Ludwig-Müller et al. (1995b). Basically, the reaction mixture contained in a total volume of 500 µl: 100 µl microsomal membranes, 2 m*M* acetyl-CoA, and 6 m*M* ATP. The reaction was started by the addition of 0.5 m*M* non-labelled IAA. N-Ethylmaleimide (NEM), dithiothreitol (DTT), *p*-chloromercuriphenylsulfonate (PCMBS), β-mercaptoethanol, H_2O_2, KCN and sodium azide were 1 m*M* in the incubation mixture; NAA, PAA, 1-IAA, 2,4-D, 3-hydroxybutyric acid, α-ketobutyric acid, and γ-guanidinobutyric acid were used at concentrations of 500 µ*M*. Tunicamycin treatment was carried out with a 120 µg ml^{-1} solution and the plant material was pre-incubated for 4 h. The enzyme reaction was carried out at pH 7.0, and incubation time was routinely 1 h at room temperature. The reaction was stopped by adding 30 µl 1 *M* HCl, and the aqueous phase was then extracted with 500 µl of ethyl acetate. The organic phase was removed, evaporated to dryness and resuspended in 20 µl methanol. The sample was kept in liquid nitrogen prior to HPLC analysis.

Protein was determined with the BCA protein assay reagent (Pierce Chemical Company, Rockford, IL, USA) using BSA as standard.

The experiments were repeated two to four times using different enzyme preparations. All results present means of independent experiments. SE was between 10–15% for the different experiments.

Extraction of endogenous IBA

The plant material was harvested after 7 days of culture in the light, extracted with 70% acetone and further purified according to Ludwig-Müller et al. (1993). The analysis of free IBA was performed by HPLC (see below).

Tab. 1. Separation of IBA synthetase containing microsomal membranes (50 000 *g* pellet) on a discontinuous sucrose gradient with subsequent marker enzyme analysis. The membranes were isolated from light grown maize (*Zea mays* L.). Enzyme activity is expressed as ng IBA (mg protein)$^{-1}$ min^{-1} for IBA synthetase, as nkat (mg protein)$^{-1}$ for all other enzymes. PM, plasma membrane; Sup., supernatant.

Enzyme	Fraction (percentage sucrose)					
	1 (0%, Sup.)	2 (18%)	3 (26%)	4 (31%)	5 (36%)	6 (45%)
IBA synthetase	–	traces	1.11	1.20	–	–
Cytochrome *c* oxidase	–	0.22	0.95	1.03	0.68	0.18
NADH-cytochrome *c* reductase (antimycin A insensitive)	–	1.29	0.61	0.79	0.27	–
ATPase, PM	–	–	–	0.06	0.02	0.01
ATPase, Golgi	–	–	–	0.02	0.03	0.02
ATPase, tonoplast	–	–	0.02	0.06	0.03	0.03
PP_i-ase	–	–	0.11	0.07	0.03	0.02
UDPase	–	0.11	0.24	0.17	0.16	0.13
Protein (mg ml^{-1})	–	1.2	4.2	7.2	13.8	27.4

HPLC analysis

The total methanol extract (20 µl, see above) was subjected to HPLC (Biotronik BT 8100), equipped with a 4.6 × 125 mm Lichrosorb C_{18} (5 µ), reverse phase column and a UV detector (at 280 nm). As solvent, 52% methanol containing 1% (v/v) acetic acid was used at a flow rate of 0.7 ml min^{-1}.

Determination of known substances was achieved by co-chromatography with authentic standards. The amount of the reaction product was determined using a standard curve with IBA.

Results

Identification of the IBA synthetase and product X-forming enzyme containing membranes

Plasma membrane-enriched fractions from light- and dark-grown maize seedlings, which contain IBA synthetase and product X-forming enzyme, respectively, were prepared by two-phase partitioning with PEG/dextran between 6.0 and 8.5% of each polymer. Very low IBA synthetase activity was found in the upper phase of all polymer concentrations, where plasma membranes accumulate. Most of the activity was found in the lower phases between 7.5 and 8.5% polymer concentration (data not shown). In contrast, product X-forming enzyme activity showed a maximum in the upper phase at 8.5% polymer concentration.

Further characterization of the IBA synthetase containing membranes was achieved by sucrose densitiy centrifugation on a step gradient between 18 and 45% sucrose and subsequent marker enzyme analysis of the respective bands (Tab. 1). IBA synthetase was mainly found at 26 and 31% sucrose, co-purifying with NADH-cytochrome *c* reductase (antimycin A insensitive) activity, which is considered as a marker for the endoplasmic reticulum (Briskin et al. 1987). Nitrate-insensitive plasma membrane ATPase was enriched between 31 and 45% sucrose and the same was found for the ATPases from other membranes. The activity of cytochrome *c* oxidase, the mitochondrial marker, was distributed in all fractions, although intact mitochondria should have been pelleted at 10 000 *g* during the preparation of the microsomal membranes. We assume that the peculiar pattern of the marker enzyme distribution (ATPases) in part results from overloading the gradient. To recover enough enzyme for measurement of IBA synthetase it was necessary to load high membrane concentrations on the gradient which resulted in not clearly resolved bands, especially in the higher density fractions of the gradient. However, the differences in the distribution of the enzyme activites are clear enough to allow a correlation of IBA synthetase activity with the ER marker NADH-cytochrome *c* reductase.

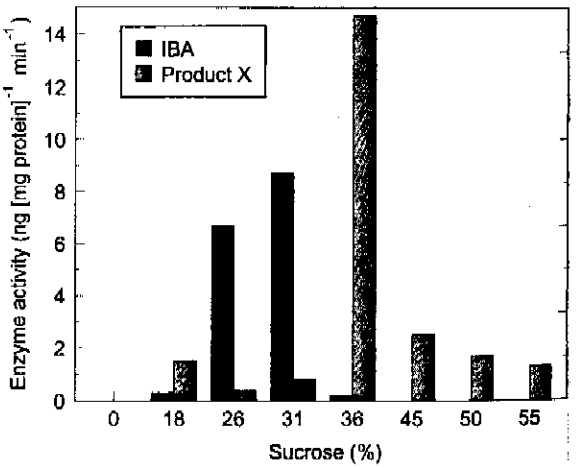
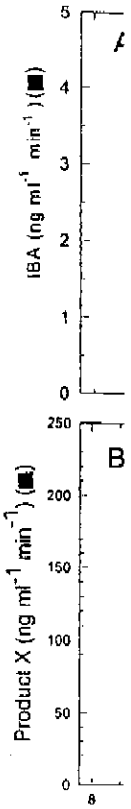


Fig. 1. Separation of IBA synthetase and product X-forming enzyme containing microsomal membranes from *Zea mays* L. on a sucrose step gradient between 18 and 55%. The individual fractions were collected and assayed for IAA conversion. (■) IBA synthetase, (▩) product X-forming enzyme.



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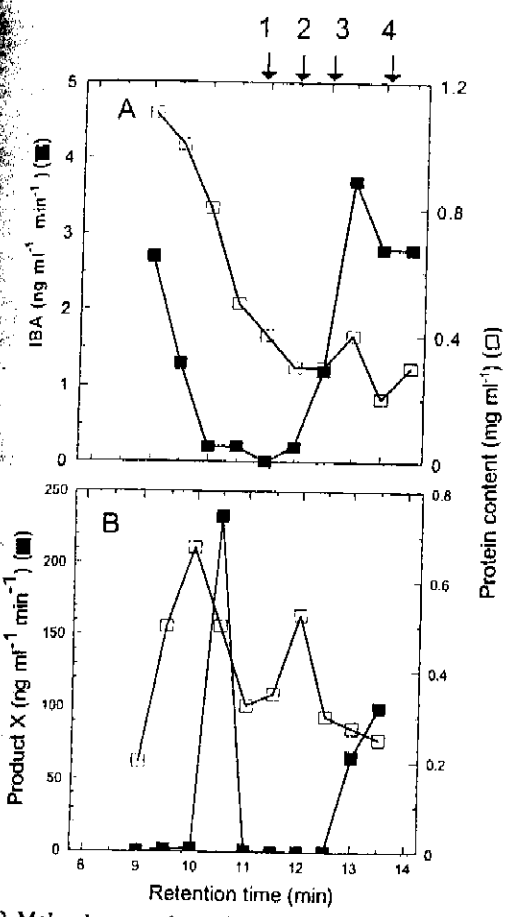

Fig. 2. Molecular mass determination of IBA synthetase (A) and product X-forming enzyme (B). IBA synthetase was extracted from light-grown maize seedlings, product X-forming enzyme from dark-grown maize seedlings. Separation of proteins was achieved on a silica gel column with bound diol phase (Zorbax GF 250). The solvent was 100 m*M* Na_2HPO_4, pH 7.0 at a flow rate of 0.8 ml min^{-1} and detection of proteins at 280 nm. The calibration of the system was done with the following standard proteins (marked by arrows): 1, BSA (66 kDa); 2, egg albumin (45 kDa); 3, pepsin (35 kDa); 4, chymotrypsinogen A (25 kDa).

Separation of microsomal membranes from dark-grown maize seedlings on the same gradient revealed the highest product X-forming enzyme activity in the 45% fraction. Therefore membranes in fractions containing both enzyme activities (from total light-grown seedlings) were separated on a gradient ranging from 18–55% sucrose. Most of the product X-forming enzyme activity was found in the 36 to 55% sucrose concentration (Fig. 1), whereas IBA synthetase was again found in the 26 to 31% fractions.

For further purification it was necessary to solubilize the respective membrane proteins. IBA synthetase was best solubilized after treatment with CHAPS or high NaCl concentrations, whereas the highest product X-forming activity was found in the supernatant after treatment with 1% (w/v) Tween 20.

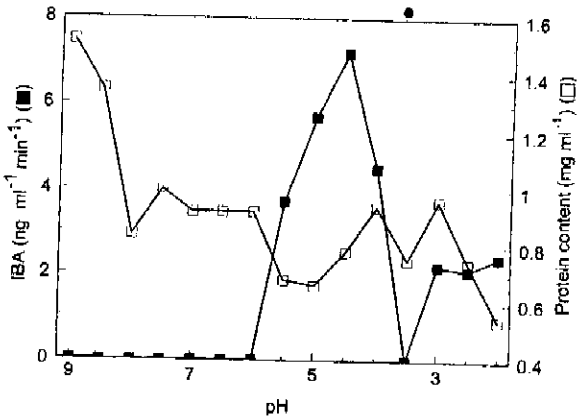

Fig. 3. Isoelectric focusing of CHAPS solubilized membranes from light-grown maize seedlings after sucrose density centrifugation. IEF was carried out using Sephadex G-75 as matrix. Single fractions were collected, centrifuged and IBA synthesis and pH value determined from the supernatant. The pI of IBA synthetase was 4.8.

Molecular mass determination of IBA synthetase and product X-forming enzyme

After separation of the CHAPS solubilized membrane proteins from light-grown maize on a gel filtration column (Zorbax GF-250) two peaks of IBA synthetase activity were detected (Fig. 2A). One peak appeared in the exclusion volume of the column and is therefore believed to contain protein detergent clusters, the second peak was found at a molecular mass of ca 31 kDa. After solubilisation with 1 *M* NaCl, an additional third peak of ca 63 kDa appeared (data not shown). Separation of solubilized product X-forming enzyme showed two activity peaks, at ca 90 and 20 kDa (Fig. 2B).

Determination of the isoelectric point of IBA synthetase

Isoelectric focusing of solubilized IBA synthetase containing membranes after sucrose densitiy gradient centrifugation revealed one major activity peak between pH 4.5 and 5.5. Maximum activity corresponded to a pI value of 4.8 (Fig. 3).

Further purification of the IBA synthetase

For further purification of IBA synthetase the active fractions after gel filtration were pooled and bound to different affinity chromatography columns. IBA synthetase could be bound to an ATP-agarose column with high affinity and it could be eluted with 50 m*M* ATP (Tab. 2). Chromatography on affinity columns with PAA and IAA showed that the enzyme bound to the column, but it could not be eluted in an active form with either IAA, NAA or PAA. Affinity chromatography on Concanavalin A-Sepharose showed that IBA synthetase did not bind to the matrix (data not shown). The total purification achieved

Tab. 2. Partial purification of IBA synthetase from shoots of light-grown maize (*Zea mays* L.) seedlings.

Purification step	Total protein (mg)	Total activity (ng IBA min^{-1})	Specific activity (ng IBA [mg protein]$^{-1}$ min^{-1})	Purification factor
Microsomal membranes	85.0	54.2	0.67	–
Sucrose density gradient centrifugation	20.5	30.8	1.50	2
Solubilisation	5.8	24.6	4.2	6
Gel filtration HPLC	0.55	15.8	28.8	43
ATP-affinity chromatography	0.04	30.7	76.7	1 150

after gel filtration and ATP-affinity chromatography was about 1 000-fold (Tab. 2).

The fractions after ATP-affinity chromatography were subjected to SDS and non-denaturing PAGE (Fig. 4) to check the purity of the enzyme preparation. Unfortunately, it was not possible to demonstrate clear bands on the SDS gel, when purified samples of light-grown seedlings were applied, but the same amount of protein gave one clear band of approximately 30 kDa on the native gel.

Effect of different chemicals on IBA synthetase

To obtain some information on the structure and mode of action of the IBA synthetase, the effect of different chemicals on the activity of IBA formation was examined. It was shown that reducing agents such as dithiothreitol and β-mercaptoethanol have no or only very little effect on the formation of IBA (Tab. 3). Sulfhydryl reagents like NEM (1 m*M*) and PCMBS (1 m*M*), which bind covalently to free SH-groups seem to promote the IBA synthetase activity. IBA synthesis was inhibited completely by 1 m*M* KCN and reduced by 59% after addition of 1 m*M* sodium azide. Preincubation with tunicamycin (120 $\mu g\ ml^{-1}$) inhibited the enzyme activity slightly (ca 25%), whereas the total protein content was reduced by about 50% after 4 h incubation time.

Acyl carrier protein (ACP), which is involved in fatty acid biosynthesis, had no promoting effect on IBA and product X synthesis. After addition of two different concentrations of ACP from *E. coli* (Sigma), the synthesis of product X and IBA was inhibited (Tab. 3). This effect was rather unspecific, because nitrate insensitive ATPase was also reduced in activity by about 50% after addition of 10 μg ACP.

Substrate analogs of IAA all inhibited the IBA synthetase activity (Tab. 4). The most potent inhibitor was the synthetic auxin NAA, where no IBA formation could be detected, followed by the structural analog 1-IAA with 92% inhibition. PAA and 2,4-D inhibited the reaction by 85 and 89%, respectively. Product analogs (substances with a butyric acid side chain) inhibited IBA formation almost completely. In some assays 0.25% ethanol was present because some of the substances had to be dissolved in ethanol. The activity of IBA synthetase was inhibited by about 30% compared to the control. However, the inhibitory effects of the substances tested were much higher than this ethanol effect.

Interestingly, it was observed that strong inhibition of IBA synthesis caused a peak, with the R_f of product X, which is normally not formed under these culture conditions.

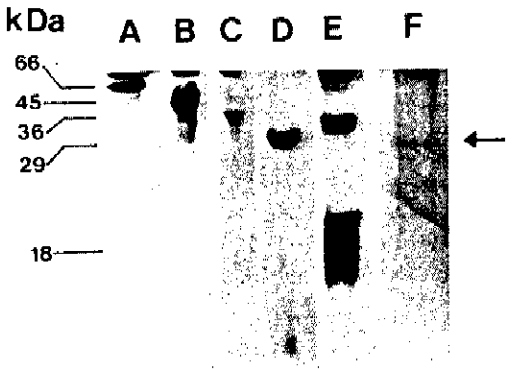

Fig. 4. Native polyacrylamide gel electrophoresis of purified IBA synthetase. The 15% gel was stained with silver nitrate. The molecular masses of marker proteins are given in the margin. Please note that an exact determination of the molecular mass is not possible on native gels. A, BSA; B, egg albumin, C, glyceraldehyde-3-phosphate dehydrogenase; D, carbonic anhydrase; E. β-lactoglobulin; F, IBA synthetase activity containing fraction after purification by gel filtration and ATP-affinity chromatography. The putative IBA synthetase band is marked by an arrow.

IBA and product X synthesis in different plant species

IBA synthetase was found in different light- and dark-grown plant species (Tab. 5) randomly chosen from different families. Three different patterns were found: (1) plants which, like maize, synthesize IBA only when they were grown in the light, whereas they form product X when cultivated in the dark, (2) plants, which were able to synthesize IBA both after growing in the light and in the dark (in the dark they also produce product X) and (3) plants, which do not synthesize IBA when light-grown, but show IBA synthetase activity when grown in the dark (these plants showed no product X formation). *Arabidopsis*, wheat and sunflower belong to the maize group (1), tobacco, Chinese cabbage, pea and carrot belong to group 2, whereas cucumber and tomato showed the characteristics of group 3. The endogenous IBA content does not correlate with any of these groups (Tab. 5). Exact values for IBA concentrations are not given, because the

Tab. 3. Effect of dif
performed with mic
addition of acyl carr
synthetase and prod
(control) was 66 mg

Condition
Control
NEM (1 m*M*)
PCMBS (1 m*M*)
β-MCE (1 m*M*)
DTT (1 m*M*)
H_2O_2 (1 m*M*)
KCN (1 m*M*)
Na-azide (1 m*M*)
Tunicamycin* (12(
ACP, light grown
1 µg
10 µg
ACP, dark grown
1 µg
10 µg

HPLC measurer
analysis and thu
dications. High c
pea and sunflow
tively), lower II
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Discussion

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Tab. 4. Effect of
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The numbers in

Substance
Control
Ethanol (0.25%)
NAA
PAA*
2,4-D*
1-IAA*
3-Hydroxybutyri
α-Ketobutyric a
γ-Guanidinobut

Tab. 3. Effect of different chemicals on the activity of IBA synthetase. All assays (with the exception of ACP addition) were only performed with microsomal membranes from light-grown maize and assayed for IBA synthetase activity. The experiment with addition of acyl carrier protein (ACP) was also performed with membranes from dark-grown seedlings and it was assayed for IBA synthetase and product X-forming enzyme activity. ND, not determined. *, The protein content in the microsomal membranes (control) was 66 mg ml^{-1}, in the tunicamycin treated membranes (4 h pre-incubation) it was reduced by about 50% to 38 mg ml^{-1}.

Condition	Activity			
	IBA (ng [mg protein]$^{-1}$ min^{-1})	% of control	Product X (ng [mg protein]$^{-1}$ min^{-1})	% of control
Control	0.65	100	0.18	100
NEM (1 m*M*)	1.15	177	ND	ND
PCMBS (1 m*M*)	0.92	141	ND	ND
β-MCE (1 m*M*)	0.53	82	ND	ND
DTT (1 m*M*)	0.70	108	ND	ND
H_2O_2 (1 m*M*)	0.58	90	ND	ND
KCN (1 m*M*)	0	0	ND	ND
Na-azide (1 m*M*)	0.38	59	ND	ND
Tunicamycin* (120 μg ml^{-1})	0.48	74	ND	ND
ACP, light grown				
1 μg	0.40	62	0	0
10 μg	0.30	46	0	0
ACP, dark grown				
1 μg	0	0	0.06	33
10 μg	0	0	0.003	1.3

HPLC measurements were not confirmed by GC-MS analysis and thus must be viewed as only relative indications. High concentrations of free IBA were found in pea and sunflower (group 2- and group 1-plants, respectively), lower IBA concentrations in maize and wheat (group 1) and Chinese cabbage and carrot (group 2). Very low IBA concentrations were found in *Arabidopsis*, tobacco and cucumber (group 1, 2 and 3, respectively) and in tomato seedlings (group 3) we could only detect traces of IBA.

Discussion

The in vitro biosynthesis of IBA from IAA has been evaluated in maize seedlings and shown that it might be a two-step reaction (Ludwig-Müller and Epstein 1992, Ludwig-Müller et al. 1995a). In the present study, IBA synthetase from light-grown maize seedlings was further characterized and partially purified. Some of the data are compared with features of product X-forming enzyme activity.

The data from the aqueous two-phase partitioning experiment and from the sucrose density gradient (Fig. 1) suggest that IBA synthetase and product X-forming enzyme activity are located on different membranes. Whereas IBA synthetase was found in the lower phase of an aqueous two-phase system, product X-forming enzyme activity was mostly detected in the upper phase, where enrichment of plasma membranes is found (Bérczi and Møller 1986, Memon et al. 1987). Marker enzyme analysis after sucrose density gradient centrifugation (Tab. 1) showed that IBA synthetase activity was correlated with antimycin A insensitive NADH-cytochome *c* reductase, a marker for endoplasmic reticulum (Briskin et al. 1987), whereas the product X-forming enzyme activity was found at higher densities, co-purifying with plasmalemma and Golgi membranes (Fig. 1).

IBA synthetase might not be an integral membrane protein, because it was possible to solubilize the activity

Tab. 4. Effect of substrate and product analogs on IBA synthetase from light-grown maize seedlings. All chemicals were 500 μ*M* in the assay (same concentration as IAA). Substances dissolved in ethanol (concentration in the assay 0.25%) are marked by an asterisk. The numbers in brackets represent % of control.

Substance	IBA synthetase (ng [mg protein]$^{-1}$ min^{-1})	Product X-forming enzyme (ng [mg protein]$^{-1}$ min^{-1})
Control	43.8 (100)	0
Ethanol (0.25%)	30.3 (69)	0
NAA	0 (0)	21.0
PAA*	6.5 (15)	0
2,4-D*	4.7 (11)	2.7
I-IAA*	3.7 (8)	2.0
3-Hydroxybutyric acid*	2.2 (5)	4.3
α-Ketobutyric acid*	1.5 (3)	5.5
γ-Guanidinobutyric acid*	2.3 (5)	18.8

Tab. 5. Determination of IBA synthetase activity in light- and dark-grown seedlings of different plants. Free endogenous IBA was measured only in light-grown plants. The IBA concentrations are not given as absolute values, because they were only determined by HPLC. (+++) High IBA concentration, (++) medium IBA concentration, (+) low IBA concentration.

Plant material	IBA synthetase activity (ng [mg protein]$^{-1}$ min^{-1})		Free endogenous IBA (light grown)
	Light grown	Dark grown	
Maize	0.52	0	++
Arabidopsis thaliana	0.48	0	+
Sunflower	0.50	0	+++
Wheat	0.93	0	++
Tobacco	0.88	5.17	+
Pea	0.58	0.33	+++
Chinese cabbage	2.17	1.50	++
Carrot	0.13	1.17	++
Cucumber	0	0.16	+
Tomato	0	0.15	traces

not only with detergents, but also with high salt concentrations. During the extraction procedure no IBA synthetase was washed from the membranes, because no activity was found in the supernatant after centrifugation at 50 000 *g* (Ludwig-Müller and Epstein 1992, Ludwig-Müller et al. 1995a).

It was shown that IBA synthetase is an acidic protein (Fig. 3) with a molecular mass of ca 31 kDa (Fig. 2). After solubilization with NaCl an additional activity peak with a molecular mass of ca 63 kDa was found. Whether IBA synthetase occurs as a multiple form, or whether the higher molecular mass form is due to a preparation artifact can not be concluded from these experiments. Product X-forming enzyme activity showed a lower molecular mass (ca 20 kDa), which suggests that it might be a part of the IBA synthetase. ACP, which has in *E. coli* a molecular mass of 10 kDa, is not the missing factor (Tab. 3).

IAA seems to be the direct precursor of IBA in vivo, but no in vivo activity has been found with NAA and PAA in *Arabidopsis* (Ludwig-Müller and Epstein 1994). Therefore, it was tested whether different substrate analogs were able to inhibit IBA synthetase (Tab. 4). All substances inhibited the reaction to a large extent, but competitive inhibition of the substrate analogs can be ruled out, since equal concentrations of the competitive substrates should cause an inhibition of ca 50%, whereas IBA synthesis was inhibited almost completely by NAA, PAA, 1-IAA, and 2,4-D. Thus, we would assume a non-competitive inhibition mechanism.

The biosynthesis of IBA might be a two-step reaction (Ludwig-Müller and Epstein 1992, Ludwig-Müller et al. 1995a). Several findings lead to this hypothesis: (1) Microsomal membranes from dark-grown maize seedlings accumulate a reaction product after feeding of IAA, which can be converted to IBA by a 10 000-*g* fraction. (2) In light-grown maize seedlings, IBA is formed directly from IAA, but under inhibitory conditions for IBA synthesis, product X accumulates (Tab. 4). (3) The molecular mass of IBA synthetase is ca 30 kDa, of product X-synthesizing enzyme ca 20 kDa. Affinity purified extracts from light-grown maize showed a band of ca 30 kDa on a

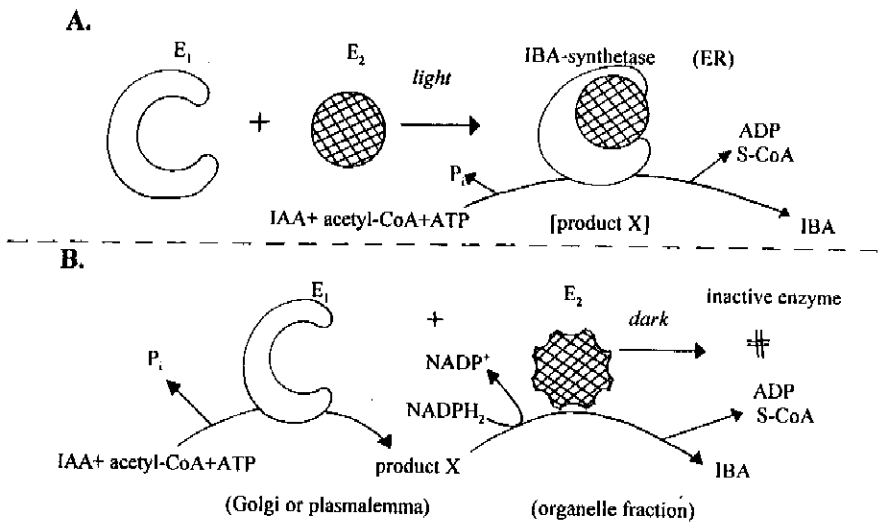

Fig. 5. Diagram on the possible distribution of IBA synthetase, product X-forming enzyme and product X-converting enzyme in light- (A) and dark-grown (B) tissue from maize seedlings. Detailed explanations are given in the discussion.

native gel (Fig. 4), whereas dark-grown extracts revealed two undistinct bands of ca 20 and 10 kDa, respectively (data not shown). Figure 5 summarizes these different findings on IBA and product X synthesis in dark- and light-grown plants. In the light (Fig. 5A), two polypeptides might form one enzyme, which most likely is located on the ER and which is able to catalyze the formation of IBA without the release of an intermediate. In the dark (Fig. 5B), one part of the IBA synthetase seems to be located on Golgi or plasma membrane vesicles. This part is able to synthesize product X using the same cofactors as IBA synthetase. A second enzyme (or a part of the IBA synthetase), which is found in an organelle fraction, can convert product X to IBA. This protein might be changed in the dark, so that it can not bind to the product X-forming enzyme. This could then lead to the accumulation of the intermediate. However, it cannot be ruled out that two totally different enzyme systems are involved in the dark and in the light, and therefore further investigation is necessary to differentiate between these hypotheses.

No essential SH-groups or disulfide linkages (Tab. 3) seem to be involved in IBA synthesis, because the reaction was not inhibited by SH- and sulfhydryl-reagents. The inhibition of IBA synthetase by sodium azide and KCN points to a metal prosthetic group as it is usually found in peroxidases (Takahama and Egashira 1991), although the nature of IBA synthetase is unlike that of the peroxidases, because no stimulation with H_2O_2 was observed (Tab. 3). Pre-treatment with tunicamycin, which has effects on the synthesis, glycosylation and transport of glycoproteins (Elbein 1987), had very little effect on IBA formation. It is thus assumed that IBA synthetase is not a glycoprotein. These results were confirmed by affinity chromatography with Concanavalin A-Sepharose, where IBA synthetase did not bind to the affinity matrix.

IBA was found as an endogenous compound in different plants and the in vivo biosynthesis has been examined in maize (Ludwig-Müller and Epstein 1991) and *Arabidopsis* (Ludwig-Müller and Epstein 1994). Further investigations on the occurrence of IBA and IBA synthetase has shown that IBA synthetase is found in different plant species, although under different culture conditions (Tab. 5). High or low IBA synthetase activity could not be correlated with the endogenous IBA content. The differences in IBA synthetase activity, when the different plants were grown under continuous illumination or in the dark might suggest that the sensitivity for light plays a role in expression of IBA synthetase. Recent findings revealed that IBA synthetase activity is enhanced by drought stress and ABA (Ludwig-Müller et al. 1995b). These results suggest that environmental (stress, light) and endogenous factors (hormones) are involved in the regulation of IBA synthesis. Purification of IBA synthetase to homogeneity will afford an opportunity to study the role of IBA more closely at the biochemical and molecular levels.

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Introduction

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Identification and quantification of three active auxins in different tissues of *Tropaeolum majus*

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Indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and phenylacetic acid (PAA) were identified as endogenous compounds with auxin activity in nasturtium (*Tropaeolum majus* L.) by full scan gas chromatography-mass spectrometry. The endogenous concentrations of the three auxins were measured by GC-selected ion monitoring-MS and isotope dilution analysis using stable labelled isotopes. PAA was present at concentrations about 10- to 100-fold lower than IAA, whereas IBA was found to be in the same concentration range as IAA. Free IAA was highest in roots followed by young leaves. IBA was also highest in the roots, and relatively high concentrations were found in young leaves and flowers. The distribution of PAA was quite different from that found for IBA. No PAA could be detected in young leaves and flowers, and in all other tissues studied the concentrations were well below those of the other two auxin compounds. The presence of a nitrilase gene family and nitrilase activity in extracts from *T. majus* suggests that PAA might be synthesized by the nitrilase pathway using benzylglucosinolate as precursor.

Introduction

Auxins are defined as growth stimulating substances, which occur in low concentrations in plant tissues. They regulate many different processes, such as cell elongation and division, induction of root growth, flower and fruit development and fruit ripening (Thimann 1977, Davies 1995). Although indole-3-acetic acid (IAA) is considered the major native auxin in higher plants and therefore also the most studied auxin (Normanly et al. 1995), other substances with auxin activity including 4-chloro-indole-3-acetic acid, indole-3-butyric acid (IBA), and phenylacetic acid (PAA), also occur in plants.

IBA was long regarded as a synthetic auxin, shown to have higher ability to promote adventitious root formation in comparison to IAA (Nordström et al. 1991). However, IBA has been demonstrated to be a natural constituent of several plant species including maize, tobacco and *Arabidopsis thaliana* (Epstein et al. 1989, Ludwig-Müller and Epstein 1991, Sutter and Cohen 1992, Epstein and Ludwig-Müller 1993, Ludwig-Müller et al. 1993) and its biosynthesis from IAA has been described (Ludwig-Müller and Epstein 1991, Ludwig-Müller and Hilgenberg 1995). PAA has been reported to occur in pea (Wightman and Lighty 1982), but possesses lower auxin-like activity than IAA (Wightman and Lighty 1982, Leuba and LeTourneau 1990) and its physiological role in vivo is not clear.

PAA is of interest, however, because it can potentially be derived from benzylglucosinolate in the glucosinolate containing species *Tropaeolum majus* (nasturtium), in a similar manner IAA is formed from indole glucosinolates in species of the Brassicaceae (Fig. 1; Ludwig-Müller 1999). *T. majus* is an annual ornamental plant with some commercial value and the flowers are edible and frequently used in salads and as decoration. *T. majus* is a unique species in which to study the relationship between auxin (IAA and PAA) and glucosinolate metabolism, since the glucosinolate pattern is much less complex compared with that found in other plant species (Bennett et al. 1996). Since *T. majus* possesses one major glucosinolate (benzylglucosinolate), it is an inter-

Abbreviations – 3-CP, 3-cyanopyridine; Cyt P450, cytochrome P450 dependent monooxygenase; GC-SIM-MS, gas chromatography-selected ion monitoring-mass spectrometry; IAN, indole-3-acetonitrile; IAOX, indole-3-acetaldoxime; IBA, indole-3-butyric acid; IMG, indole-3-methylglucosinolate; NIT, nitrilase; PAA, phenylacetic acid; POD, peroxidase.

esting plant species to study glucosinolates and related auxins during different developmental processes, but also during pathogenesis and symbiosis. It was recently shown that *T. majus* could be successfully inoculated with the causal agent of clubroot disease, *Plasmodiophora brassicae* (Ludwig-Müller et al. 1999). Inoculation with different *Glomus* species and *Gigaspora* resulted in a functional arbuscular mycorrhizal relationship (Vierheilig et al. 2000). Our interest is in the role auxins play in the interaction of *T. majus* with associated microorganisms (Lambrecht et al. 2000). *T. majus* is unique with respect to the possibility to compare for example pathogenesis (clubroot) and symbiosis (arbuscular mycorrhiza) in a glucosinolate containing species.

The biosynthesis of auxins can proceed via different pathways involving tryptophan or indole as a precursor (Normanly et al. 1995). The biosynthetic pathways for auxins in *T. majus* have not been investigated, but an attractive hypothesis is that *T. majus* has a pathway using a nitrile intermediate common with that found in the Brassicaceae (Ludwig-Müller and Hilgenberg 1988) and *Arabidopsis* (Ludwig-Müller and Hilgenberg 1992, Hull et al. 2000), since all are glucosinolate containing plant species (Bennett et al. 1996, Ludwig-Müller et al. 1999). The major glucosinolate in *T. majus* is benzylglucosinolate (or glucotropaeolin; Bennett et al. 1996, Ludwig-Müller et al. 1999), but other glucosinolates have been identified as minor components (Vierheilig et al. 2000). The glucosinolate/IAA biosynthetic pathway in Brassicaceae involves the conversion of tryptophan to indole-3-acetaldoxime (IAOX; Ludwig-Müller and Hilgenberg 1988, Ludwig-Müller et al. 1990, Hull et al. 2000), indole-3-methylglucosinolate (IMG; Helmlinger et al. 1985) and indole-3-acetonitrile (IAN; Ludwig-Müller and Hilgenberg 1990, Normanly et al., 1993). In *T. majus* a Cyt P450 dependent enzyme activity has been isolated from microsomal membranes that is likely to be involved in the conversion of phenylalanine to the respective oxime (Du and Halkier 1996). The glucosinolates and the enzyme myrosinase (β-thioglucoside glucohydrolase, EC 3.2.3.1) are localized in different compartments (Bones and Rossiter 1996). Upon tissue dis-

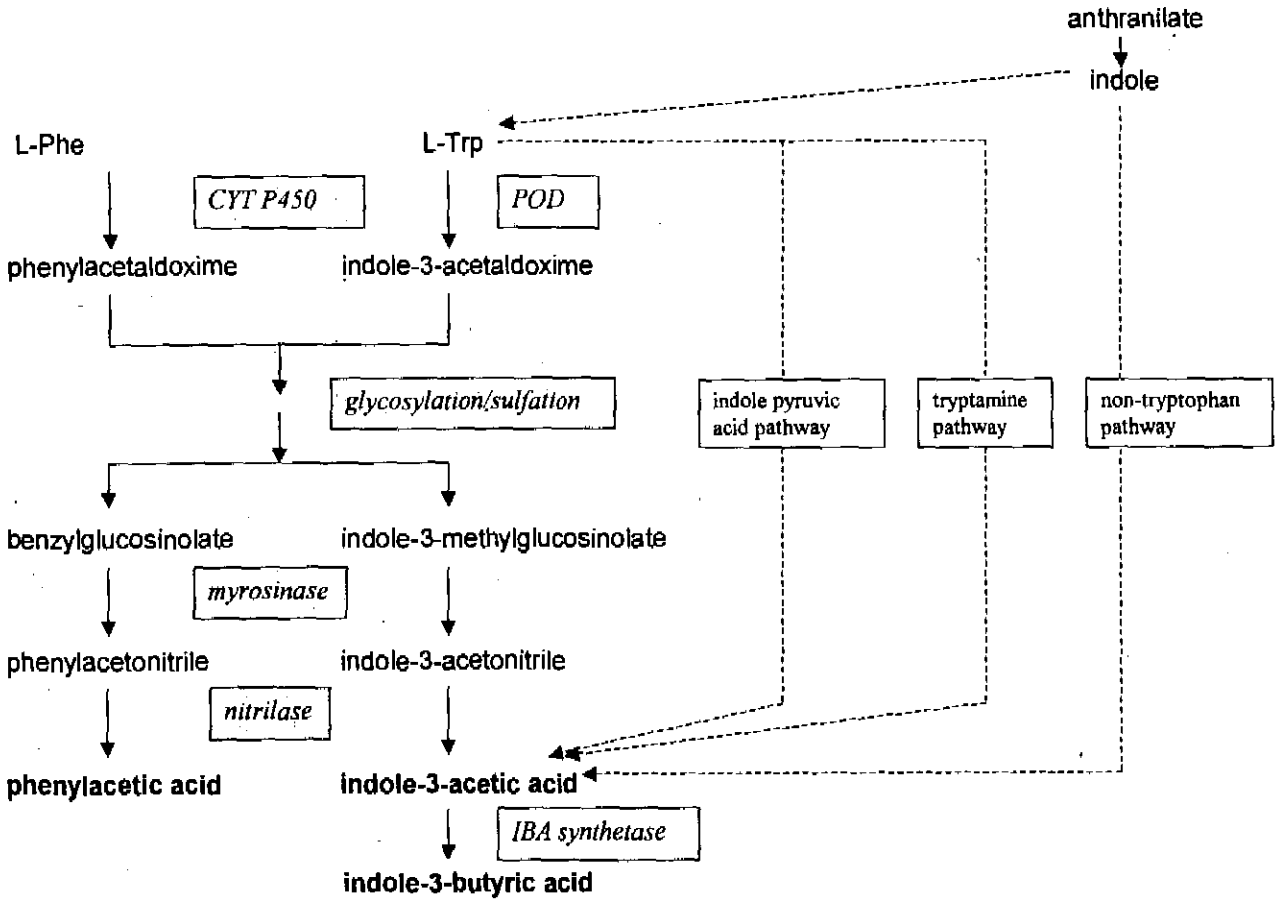

Fig. 1. Hypothetical biosynthetic pathways for the three different auxins identified as natural constituents of *T. majus*. Reactions are proposed on the basis of what has been hypothesized for other plant species. The presence of both myrosinase (Lykkesfeld and Møller 1993) and nitrilase (this paper) has been demonstrated in *T. majus*. Cyt P450; cytochrome P450-dependent monooxygenase(s), catalysing the conversion of L-Trp to indole-3-acetaldoxime in *Arabidopsis thaliana* (Hull et al. 2000) and of L-Phe to phenylacetaldoxime in *T. majus* (Du and Halkier 1996). The latter reaction is catalysed in Brassicaceae by flavin containing monooxygenases (Bennett et al. 1996). Alternatively, L-Trp can be converted to indole-3-acetaldoxime in *Arabidopsis* and Brassica by basic peroxidase isoenzymes (POD; Ludwig-Müller and Hilgenberg 1988, 1992, Ludwig-Müller et al., 1990).

ruption, glucosinolates can be converted by myrosinase to different products, among them a nitrile. In the case of IMG this would lead to increased levels of the auxin precursor IAN. A 'shortcut' of this pathway, which might operate in undamaged tissue, does not involve synthesis of IMG, instead IAOX is directly converted to IAN (Ludwig-Müller and Hilgenberg 1990). IAN seems to play a key role in at least one of the major pathways for the biosynthesis of IAA in Brassicaceae. The enzyme nitrilase (nitrile aminohydrolase, EC.3.5.5.1), which converts IAN to IAA, might therefore be important for regulation of IAA biosynthesis in this plant family (Normanly et al. 1993). Several nitrilase (Bartel and Fink 1994, Bartling et al. 1994, Bischoff et al. 1995) and myrosinase (Xue et al. 1992, 1995) genes have been cloned from *Brassica* species and *Arabidopsis thaliana*.

An analogous pathway for the biosynthesis of PAA can be proposed, since the conversion of benzylglucosinolate by myrosinase and nitrilase would result in phenylacetonitrile and subsequently in PAA. However, other biosynthetic pathways for IAA (and also PAA) can not be ruled out and it seems likely that multiple pathways to IAA exist in a number, if not all, plant species (Bartel 1997, Sitbon et al. 2000). Although present in plant tissues, the biosynthesis of IBA has yet to be demonstrated by precursor labelling studies in *T. majus*. Putative pathways for IAA, PAA and IBA biosynthesis in *T. majus*, based on results obtained in related plant species, are summarized in Fig. 1. Alternative IAA biosynthesis pathways include indole-3-pyruvic acid (Tam and Normanly 1998) and tryptamine (Zhao et al. 2001) as intermediates. We now demonstrate the occurrence of three different auxins, two of them (IAA and PAA) related to glucosinolate biosynthesis (see Reintanz et al. 2001), in different tissues of *T. majus* and report the endogenous concentrations of the three auxins in specific parts of the plant.

Materials and methods

Plant material

T. majus L. cv. nanum (Dehner Gartencentre, Rain am Lech, Germany) seeds were sown onto compost and cultivated in a greenhouse at 23°C, 60% humidity and with a 16-h light/8-h dark cycle. The plants were harvested 3 weeks after germination and divided into different tissues (see Table 1). Flowers were harvested from mature plants at bloom. Tissues were frozen at harvest in liquid N_2 and stored at −80°C prior to analysis.

For determination of auxin responsiveness, plants (*T. majus* L. cv. nanum and *Zea mays* L. cv. Lixis) were grown on filter paper soaked with distilled water and the seedlings harvested after 7 days of culture in the dark.

Auxin extraction and purification

Identification of endogenous auxins. The frozen plant material (100 g FW of root, leaf and shoot tissue) was pulverized in liquid N_2, then extracted with 65% 2-propanol in 200 m*M* imidazole buffer, pH 7 on ice. The extracts were filtered, evaporated to the aqueous phase and then centrifuged. The supernatant was brought to pH 7 and extracted three times with ethyl acetate. The aqueous phase was then adjusted to pH 3 and extracted again three times with equal volumes of ethyl acetate. The organic fractions were pooled, evaporated to dryness and the sample resuspended in 65% 2-propanol in 200 m*M* imidazole buffer, pH 7. The sample was diluted 1 : 10 with distilled water and subjected to purification on a NH_2-column as described by Chen et al. (1988). Further purification of IAA, IBA and PAA was performed by HPLC (Biotronik BT 8100, 63477 Maintal, Germany). The solvents (1% acetic acid in water [A] and methanol [B]) were administered as a linear gradient from 30% to 60% B (0–20 min), and elution was continued for an additional 10 min with 60% B. Flow rate was 0.7 ml min^{-1}. Chromatography of authentic standards with detection at 280 nm allowed determination of the retention times for IAA/PAA at 15.5 min and for IBA at 22.7 min. The compounds were collected by retention time and evaporated separately to dryness. The IBA containing fraction was methylated (Cohen 1984) and then resuspended in ethyl acetate. Since it was not possible to separate all three auxins from each other in one run, the IAA/PAA containing peak was collected, evaporated to dryness, resuspended in a small amount of methanol and subjected to a second HPLC separation. Separation was performed using an isocratic system with 65% A/ 35% B and a flow rate of 0.7 ml min^{-1}. Under these chromatographic conditions PAA eluted at a retention time of 15.3 min and IAA at 16.9 min. The peaks were collected separately and evaporated to dryness. The extracts were methylated with diazomethane (Cohen 1984) and resuspended in ethyl acetate. Methylated samples were kept at −80°C and shipped by express carrier on dry ice. The methylated extracts were then injected directly into the GC-MS.

Quantification of endogenous free auxins. The frozen plant material (approximately 1 g FW per analysis) was extracted and purified as described above, with the ex-

Table 1. Endogenous concentrations of free indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and phenylacetic acid (PAA) in different tissues of *T. majus*. The numbers in parenthesis refer to the tissues as given in Fig. 3. The endogenous concentrations were determined by isotope dilution analysis after analysing the samples by GC-MS. Each value represents the mean ± SE of three independently extracted samples.

	Endogenous free auxin (ng g^{-1} FW)		
Tissue	IAA	IBA	PAA
Root (1)	119 ± 26	61 ± 26	1.6 ± 0.2
Hypocotyl (2)	29 ± 7	21 ± 7	1.9 ± 0.3
Shoot (3)	18 ± 3	16 ± 6	1.6 ± 0.03
Leaf stalk (4)	13 ± 3	11 ± 5	1.8 ± 0.1
Older leaf (5)	15 ± 2	22 ± 5	1.5 ± 0.2
Young leaf (6)	55 ± 12	30 ± 4	< detection limit
Flower (7)	12 ± 0.5	31 ± 9	< detection limit

ception that the neutral/acidic ethyl acetate fractionations were omitted and the extracts were directly purified on NH_2-columns (Chen et al. 1988). To each sample 200 ng $^{13}C_6$-IAA (Cambridge Isotope Laboratories, Andover, MS, USA), 100 ng $^{13}C_1$-IBA (Sutter and Cohen 1992) and 100 ng $^{13}C_1$-PAA (Cambridge Isotope Laboratories, Andover, MA, USA) were added. For each sample three independent extractions were performed. HPLC purification and methylation of samples was as described above.

GC-MS analysis

Analysis was performed by GC-MS on a Hewlett Packard 6890 GC/5973 MSD system. IAA and IBA were separated on a Durabond-5 column, 30 m × 0.25 mm, 0.25 µm film. The temperature program was 70°C for 2 min, followed by an increase of 20° min^{-1} − 280°, and kept at this temperature for 5 min. IAA and IBA eluted under these conditions at 10.1 and 11.2 min, respectively. PAA was chromatographed on the same column, but with some modifications in the temperature program (initial temperature 70°C for 2 min, 5°C min^{-1} to 180°C, 20°C min^{-1} to 280°C, 280°C for 5 min). Under those conditions PAA eluted at 9.7 min. Full scan spectra were recorded for the peaks co-chromatographing with authentic methylated standards and the mass spectra compared to spectra obtained from authentic standards.

The endogenous concentrations of free auxins were calculated by the isotope dilution equation as given by Ilic et al. (1996). For the determination of free IAA, the molecular and quinolinium ions of the methylated substance at m/z 189/195 and 130/136, respectively, were monitored (ions deriving from endogenous and $^{13}C_6$-IAA), for free IBA the molecular and quinolinium ions at m/z 217/218 and 130/131, respectively (ions deriving from endogenous and $^{13}C_1$-IBA), and for PAA the molecular and tropylium ions at m/z 150/151 and 91/92, respectively (ions deriving from endogenous and $^{13}C_1$-PAA).

Growth assay

From the hypocotyls and roots of dark-grown *T. majus* seedlings, 0.5 cm segments (hypocotyl segments directly below the cotyledons, root segments 3 mm away from root tip) were excised under green safety light with two razor blades mounted on a handle exactly 5 mm apart and placed into Petri dishes containing the respective auxin at concentrations of 0, 1, 10, 100 and 1000 µ*M* in a solution of 50 m*M* sucrose, 10 m*M* KH_2PO_4, 1 m*M* $MgSO_4$, 0.5 m*M* $Ca(NO3)_2$, 0.1 m*M* $FeCl_3$, pH 6.2. Shoot segments from dark-grown *Z. mays* seedlings were cut 3 mm below the coleoptile tip and treated likewise. In one series, in addition to IAA, a brassinosteroid (BS; 22α,23α-dihydroxy-24β-methyl isomer of brassinolide) was added (Cohen and Meudt 1983). The plant segments were incubated 24 h in the dark at 23°C and then the length of the segments was measured using a binocular microscope (10-fold magnification).

IAA degradation assay

The same conditions as described for the growth assay (buffer, incubation in the dark for 24 h at 23°C) were used to determine the degradation of IAA by segments of *T. majus* and *Z. mays*. Initial IAA concentration in 10 ml solution was 3 µg. For each condition, 20 5-mm segments (hypocotyls for *T. majus* and coleoptiles for *Z. mays*) were used and the experiment was performed in three replicates. The control solution was incubated under the same conditions without plant segments. After 24 h the segments were removed, the medium brought to pH 3 and twice extracted with ethyl acetate. The organic phases were combined, evaporated to dryness and resuspended in 20 µl 100% methanol, which was subjected completely to HPLC analysis (conditions as described above). The amount in the control solution was set as 100% (recovery from the control solution of IAA was 80%). The data were normalized on segment number.

DNA gel blot analysis

Extraction of genomic DNA was performed according to Murray and Thompson (1980) and quality was checked by agarose gel electrophoresis. Genomic DNA was digested with *Eco*RI, separated on a 0.8% agarose gel, blotted onto nylon membrane and hybridized with a biotinylated cDNA probe against nitrilase 1 from *Arabidopsis thaliana* (Bischoff et al. 1995). Non-radioactive Southern blots were performed with biotinylated probes prepared by PCR according to Löw and Rausch (1994), with the Southern-Light™-kit from Tropix (Serva) for detection.

Nitrilase extraction and determination of enzyme activity

Light- or dark-grown seedlings (roots, leaves, hypocotyls were harvested separately) were harvested and extracted with a 0.1-*M* Tris-HCl-buffer, pH 7.5 containing 1 m*M* EDTA and 0.2 m*M* β-mercaptoethanol (Rausch and Hilgenberg 1980). The homogenate was filtered and the filtrate was centrifuged for 60 min at 50 000 *g*. The supernatant (cytoplasmic fraction) was desalted over a Sephadex-G 25 column equilibrated with elution buffer (0.05 *M* Tris-HCl-buffer, pH 7.5 containing 1 m*M* EDTA and 0.04 m*M* β-mercaptoethanol). The desalted supernatant was used for determination of nitrilase activity.

Nitrilase activity using 3-cyanopyridine (3-CP) as substrate was measured photometrically by determining the release of NH_4^+ ions according to Rausch and Hilgenberg (1980). Protein was determined according to Peterson (1977) using bovine serum albumin as standard.

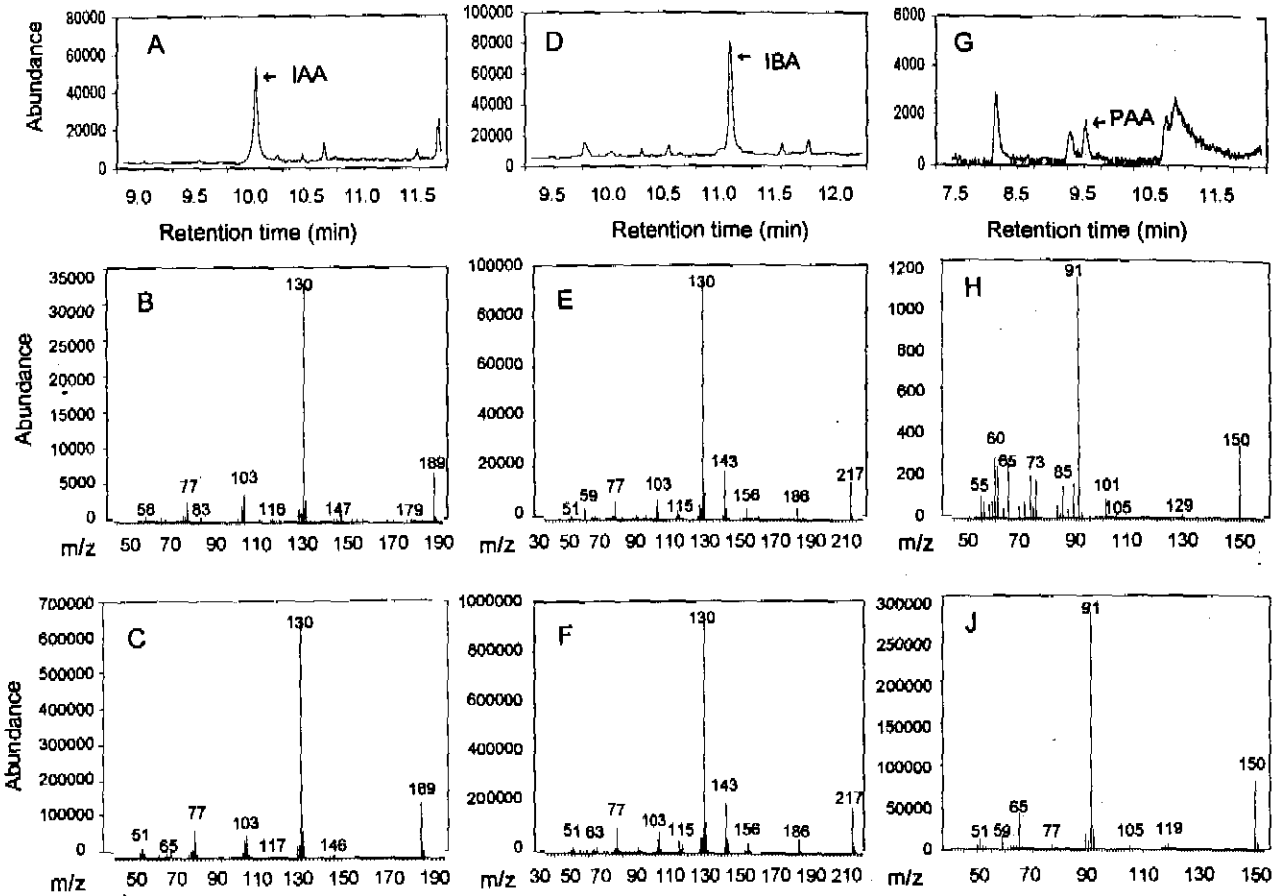

Fig. 2. Identification of IAA, IBA and PAA in *T. majus* by full scan gas chromatography-mass spectrometry. The upper traces of the respective figures represent the reconstructed ion chromatograms for HPLC-purified plant extracts from *T. majus* (A, D, G), the middle panels present the full scan spectra of the respective auxin peaks indicated by an arrow (B, E, H). In the lower panels the respective standard spectra of methylated auxins are presented. (A-C) Chromatogram and spectrum of methylated IAA (Rt 10.06–10.13 min) with the characteristic ions at m/z 189 (molecular ion), m/z 130 (quinolinium ion), m/z 103, and m/z 77. (D-F) Chromatogram and spectrum of methylated IBA (Rt 11.05–11.08 min) with the characteristic ions at m/z 217 (molecular ion), m/z 143, m/z 130 (quinolinium ion), m/z 103, and m/z 77. (G-I) Chromatogram and spectrum of methylated PAA (Rt 9.7–9.78 min) with the characteristic ions at m/z 150 (molecular ion), m/z 91 (tropylium ion), and m/z 65.

Peroxidase extraction and assay

A crude enzyme extract was prepared by homogenizing the segments with 50 m*M* Tricine, pH 7.0, containing 0.5 *M* NaCl and 15 m*M* CHAPS (Ludwig-Müller and Hilgenberg 1992). The homogenate was centrifuged for 20 min at 50 000 *g* and the supernatant directly used for peroxidase activity measurement.

Peroxidase activity was measured with *o*-phenylenediamine (OPD) as substrate according to Ludwig-Müller and Hilgenberg (1992). Briefly, one tablet of OPD (Diagnostic Product Corporation, Los Angeles, CA, USA) was dissolved in 5 ml substrate buffer (50 m*M* Tricine, pH 7 containing 3.6% H_2O_2) and incubated with 0.03–0.05 mg protein for 15 min at RT. The reaction was stopped with an equal volume of 0.5 *M* H_2SO_4 and absorbance was measured at 490 nm. A calibration curve was performed for calculation of enzyme activity using known amounts of horseradish peroxidase (Merck, Darmstadt, Germany). Protein concentrations were determined as described above.

Results and discussion

Identification of three different auxins in *Tropaeolum majus*

We were interested in the presence of different auxin-like substances within *T. majus* because of our interest in auxin involvement in plant–microbe interactions and the relative simplicity of the glucosinolate pattern of this species, which makes it an excellent model system for further studies.

After purification and analysis of the methylated extracts, it was possible to identify IAA, IBA and PAA as endogenous compounds of *T. majus* by full scan mass spectra (Fig. 2). The spectrum of methylated IAA showed the characteristic ions at m/z 189 (molecular

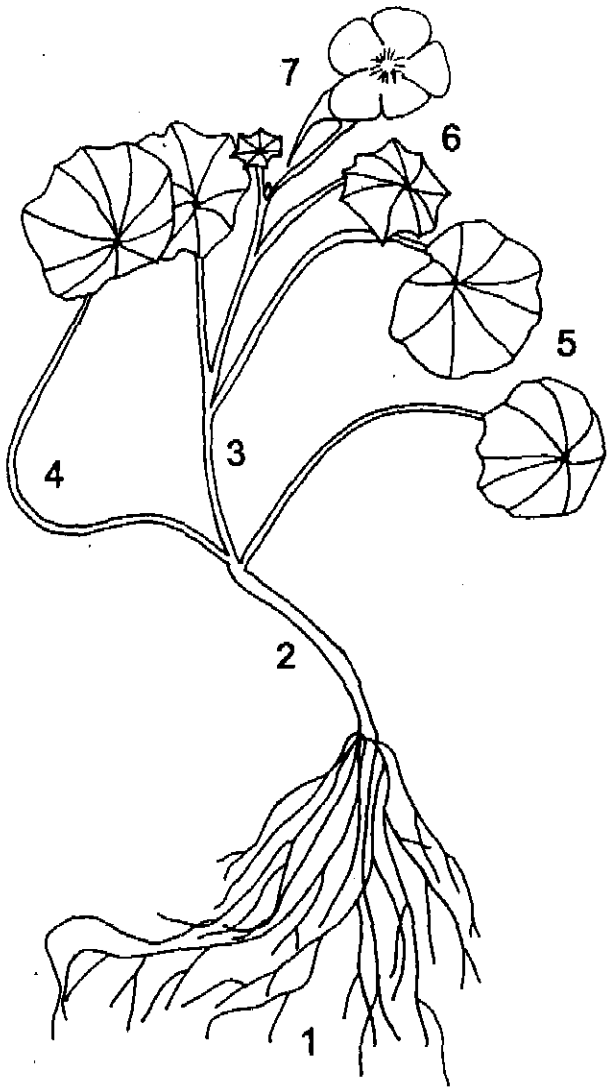

Fig. 3. Nasturtium (*T. majus*) plant with the tissues used in this study indicated and numbered. The numbers refer to those given in Table 1.

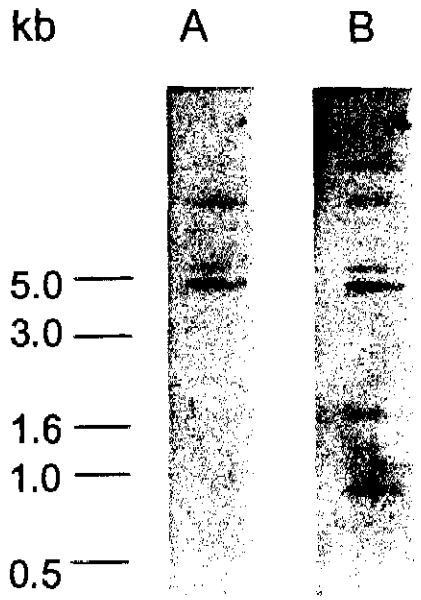

Fig. 4. Southern blot analysis of genomic DNA from *T. majus* digested with *Eco*RI. Hybridization was performed with an *Arabidopsis thaliana* nitrilase 1 cDNA probe (Bischoff et al. 1995) at 48°C. (A) High stringency wash at 60°C; (B) High stringency wash at 48°C.

ion), m/z 130 (quinolinium ion), m/z 103, and m/z 77 (e.g. McDougall and Hillman 1978, Miller et al. 1987, Slovin and Cohen 1988), the spectrum of methylated IBA the characteristic ions at m/z 217 (molecular ion), m/z 143, m/z 130 (quinolinium ion), m/z 103, and m/z 77 (e.g. Chen et al. 1988, Ludwig-Müller and Epstein 1991, Ludwig-Müller et al. 1993) and the spectrum of methylated PAA the characteristic ions at m/z 150 (molecular ion), m/z 91 (tropylium ion), and m/z 65 (Williams et al. 1969, Wightman and Lighty 1982).

Quantification of different auxins from *Tropaeolum majus*

After our identification of the three different auxins in *T. majus*, we analysed the distribution of the substances in the plant. *T. majus* plants were dissected and seven different samples (Fig. 3) were analysed for endogenous auxins using stable isotope analysis to determine endogenous levels. All three auxins were present in most tissues studied, although the concentrations were different in specific tissues (Table 1). The highest concentrations of free IAA were found in the roots, followed by young leaves and hypocotyls. The concentrations in the other tissues were similar to each other. Free IBA was also present in relatively high amounts in the roots. The concentration of PAA was about 10- to 100-fold lower than that of IAA, whereas IBA was in about the same general concentration range as IAA. PAA was below detection limit in flowers and young leaves, and in those tissues where it was found the concentrations of PAA were quite low (between 1.5 and 1.9 ng g^{-1} FW).

Nitrilase is present and active in *Tropaeolum majus*

The biosynthesis pathways for auxins in plants, including *T. majus*, have not been fully elucidated. IAA may be derived via several different pathways (Fig. 1), and one likely candidate would be the indole glucosinolate pathway demonstrated for Brassica species (Ludwig-Müller 1999, Reintanz et al. 2001). PAA may also be derived by an analogous pathway. The presence of myrosinase, critical for the conversion of indolic and benzylic glucosinolates to their respective auxins, has already been shown in *T. majus* (Lykkesfeld and Møller 1993). The last step in the biosynthesis of IAA/PAA via the glucosinolate pathway appears to be catalysed by a specific isozyme of nitrilase in various Brassicaceae (Rausch and Hilgenberg 1980, Bestwick et al. 1993, Grsic et al.

Table 2. Nitrilase activity in 12-day-old seedlings of *T. majus* measured with 3-cyanopyridine as substrate.

Growth conditions	Tissue	Nitrilase activity (pkat mg^{-1} protein)
Light	leaves	0.9
	hypocotyls	1.1
	roots	2.1
Dark	whole seedlings	2.2

1998). Four different nitrilase genes have been cloned from *A. thaliana* (Bartel and Fink 1994) and at least three different genes are present in Chinese cabbage (Bischoff et al. 1995, Grsic et al. 1999). Hybridization of restricted genomic DNA from *T. majus* with a 600-bp probe against *NIT1* from *Arabidopsis* resulted in the detection of three bands when high stringency wash was performed at 60°C (Fig. 4A). At lower stringency (48°C high stringency wash) seven bands hybridized with the *NIT1* cDNA probe (Fig. 4B), indicating the presence of a multigene family of nitrilases in *T. majus* with homology to *NIT1* from *Arabidopsis*.

In tobacco, a nitrilase-like gene was found, which showed high sequence similarity to *NIT4* from *Arabidopsis* (Tsunoda and Yamaguchi 1996), although no nitrilase activity could be detected in tobacco (Schmidt et al. 1996) suggesting that sequence homology alone is inadequate to predict function in this gene family. Therefore, the activity of nitrilase was directly measured in *T. majus* and it was found that both, light- and dark-grown seedlings of *T. majus* contained nitrilase activity (Table 2). In light-grown seedlings, the activity was highest in roots, followed by hypocotyls and then leaves. Since the activity was determined with an artificial substrate (3-CP), it can not be concluded that the putative natural substrates indole-3-acetonitrile or phenylacetonitrile are also acceptable substrates for the enzyme(s). The more detailed investigation of the substrate specificities of nitrilase(s) present in *T. majus* will be the subject of further studies, once different nitrilase cDNAs have been cloned.

IAA, IBA and PAA promote growth in *Tropaeolum majus*

We have identified IAA, IBA and PAA as endogenous substances in *T. majus* and thus it was important to show that there are tissues of *T. majus* which respond to each of the three substances. The growth promoting effect of IAA, IBA and PAA was tested on hypocotyl and root segments at different concentrations (Fig. 5). IBA showed a growth promoting effect over a wide range of concentrations on both hypocotyls and roots, whereas elongation of hypocotyls after IAA treatment showed a very sharp optimum at 1 μM. IAA promoted root growth only to a small extent. PAA increased hypocotyl and root elongation only at higher concentrations (1000 and 100 μM, respectively), which is in contrast to the low PAA concentrations found in all tissues of *T. majus*

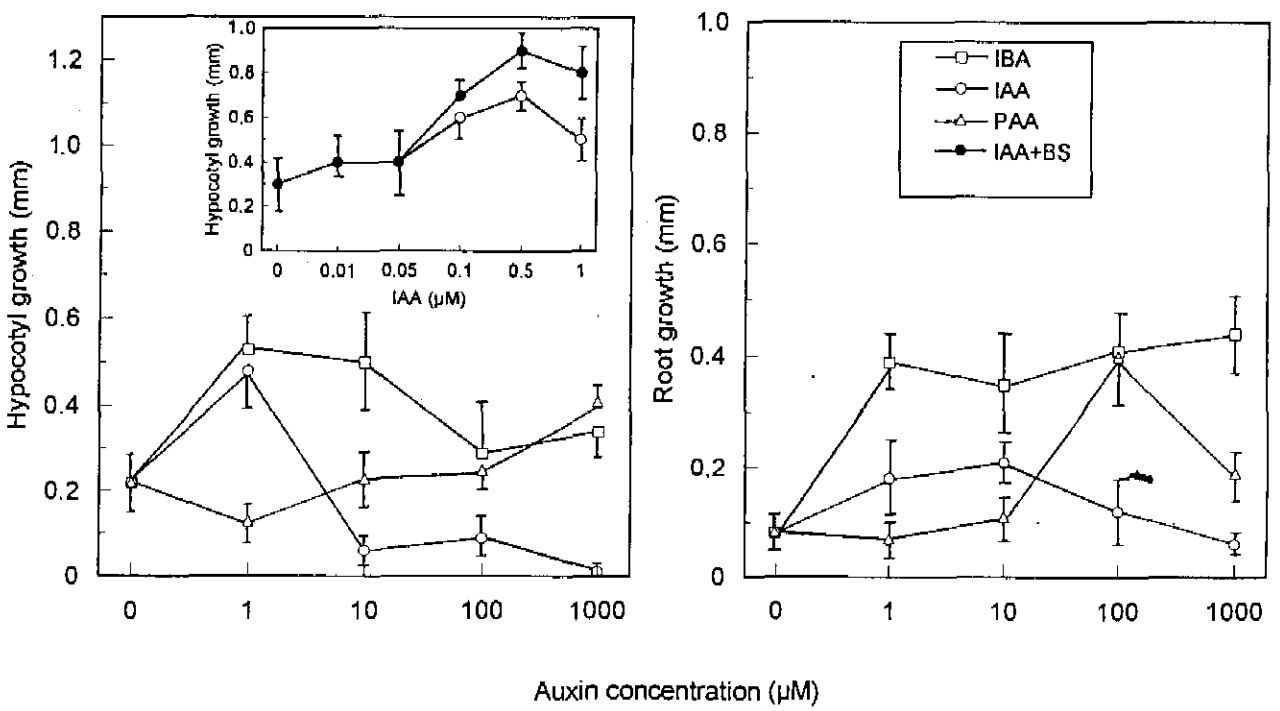

Fig. 5. Effect of IAA, IBA and PAA on the growth of hypocotyl and root segments of *T. majus* incubated for 24 h in the dark with different concentrations of the respective auxins. The inset shows the effect of low IAA concentrations in combination with different concentrations of the 22α,23α-dihydroxy-24β-methyl isomer of brassinolide (BS).

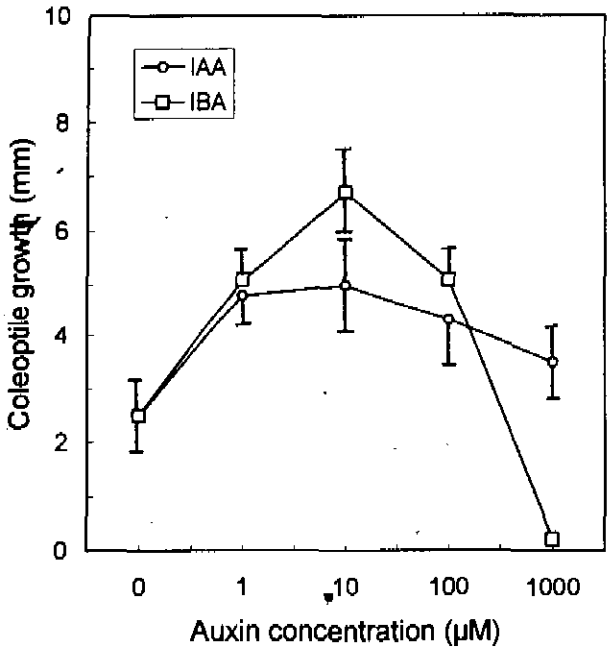

Fig. 6. Effect of IAA and IBA on the growth of coleoptile segments of *Zea mays* incubated for 24 h in the dark with different concentrations of the respective auxins.

(Table 1). However, the growth response to exogenous compounds may not fully reflect the in vivo situation.

Another possibility is that other growth promoting factors are active in *T. majus*. Cohen and Meudt (1983) have shown that simultaneous treatment with IAA and a brassinosteroid (the 22α,23α-dihydroxy-24β-methyl isomer of brassinolide; BS) gave better growth stimulating effects than one of the compounds alone. Epibrassinolide acted additively and not synergistically on the elongation growth of maize coleoptiles (Park et al. 2001). Therefore, the effect of 1 µ*M* BS was tested on IAA-induced hypocotyl elongation (Fig. 5, inset). Indeed, the addition of BS further promoted hypocotyl elongation at low IAA concentrations (optimum IAA 0.5 µ*M*), although only by about 1.3-fold.

In comparison, maize coleoptiles show a much higher growth response to IAA and IBA compared with *T. majus* under the same assay conditions (Fig. 6). IBA seems to be the slightly better growth promoting substance at lower concentrations (10 µ*M*), but it is more inhibitory at higher concentrations (1000 µ*M*).

Why is *T. majus* not responding with elongation growth in a similar manner as for example maize coleoptiles? First, the sensitivity of the tissue might be lower, thus the reaction is not as pronounced in *T. majus*, and second, the plant species may contain high peroxidase (POD)/IAA-oxidase activities, which rapidly destroy the supplied auxins. This hypothesis was tested by measuring (1) POD activity in segments of *T. majus* and for comparison in maize and (2) free IAA levels in the medium after 24 h incubation with segments from both plant species (Table 3). Higher POD activity in the segments of *T. majus* correlated with lower free IAA concentration in the medium after 24 h incubation time. While about 70% of the IAA in the medium was extractable after 24 h from the dishes containing maize coleoptile segments, only 24% of the IAA initially added was left in the Petri dishes containing *T. majus* hypocotyl segments. These results indicate that at least part of the lower growth response of *T. majus* compared to maize may be due to the higher degradation rate of IAA during the incubation period.

Table 3. Peroxidase (POD) activity in hypocotyls and coleoptile segments of *T. majus* and *Zea mays*, respectively, and remaining free IAA content in the liquid medium after incubation of *T. majus* and *Zea mays* segments for 24 h at room temperature. Means ± SE of three different experiments are given. Control solution = extraction of IAA from solution without plant material after the same incubation time.

		Remaining free IAA in solution after 24 h	
POD activity Plant species	(nkat mg^{-1}protein)	(µg)	(%)
Tropaeolum majus	44.5 ± 2.8	0.57 ± 0.09	24
Zea mays	29.8 ± 4.2	1.61 ± 0.48	69
Control solution		2.33 ± 0.41	100

Conclusion

In conclusion, we have shown that three different auxins are present in *T. majus* and that the plant can respond to all three substances in a growth assay. The presence of myrosinase, nitrilase and suitable precursor substances suggest that the biosynthesis of IAA and PAA could proceed via the glucosinolate pathway in this plant. The biosynthesis of IBA has not been studied in detail in dicot species. However, in maize IBA is produced by the enzyme IBA synthetase (Ludwig-Müller and Hilgenberg 1995). Future studies in our laboratory will focus on the biosynthesis of IBA in *T. majus* and *Arabidopsis*. The identification of three different auxins in *T. majus* is prerequisite to investigations of glucosinolate/auxin regulation during colonization by pathogenic and beneficial microorganisms, and should allow a more complete picture of the role of active auxins as reciprocal signals in such plant–microbe interactions.

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Occurrence and *in Vivo* Biosynthesis of Indole-3-Butyric Acid in Corn (*Zea mays* L.)[1]

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ABSTRACT

Indole-3-butyric acid (IBA) was identified as an endogenous compound in leaves and roots of maize (*Zea mays* L.) var Inrakorn by thin layer chromatography, high-performance liquid chromatography, and gas chromatography-mass spectrometry. Its presence was also confirmed in the variety Hazera 224. Indole-3-acetic acid (IAA) was metabolized to IBA *in vivo* by seedlings of the two maize varieties. The reaction product was identified by thin layer chromatography, high performance liquid chromatography, and gas chromatography-mass spectrometry after incubating the corn seedlings with [^{14}C]IAA and [$^{13}C_6$]IAA. The *in vivo* conversion of IAA to IBA and the characteristics of IBA formation in two different maize varieties of *Zea mays* L. (Hazera 224 and Inrakorn) were investigated. IBA-forming activity was examined in the roots, leaves, and coleoptiles of both maize varieties. Whereas in the variety Hazera 224, IBA was formed mostly in the leaves, in the variety Inrakorn, IBA synthesis was detected in the roots as well as in the leaves. A time course study of IBA formation showed that maximum activity was reached in Inrakorn after 1 hour and in Hazera after 2 hours. The pH optimum for the uptake of IAA was 6.0, and that for IBA formation was 7.0. The K_m value for IBA formation was 17 micromolar for Inrakorn and 25 micromolar for Hazera 224. The results are discussed with respect to the possible functions of IBA in the plant.

The use of natural and synthetic auxins in horticultural practice is very common, and the ability of auxins to stimulate root formation is well known (16, 20). IBA[2] is the most widely used auxin for rooting purposes in agriculture (16), and although many reports deal with its use in root propagation (16), no attention has yet been paid to its biosynthesis. Several studies provided evidence for the occurrence of IBA as a natural constituent of plants (2, 6, 12–14, 24), and there are some studies on its metabolism. Epstein and Lavee (10) showed that IBA may be converted to IAA in cuttings of grapevine and olive. Andreae and Good (1) reported that IBA-treated tissues accumulated substances that were tentatively identified as indolebutyramide and IBAsp. Chromatographic data provided by Fawcett *et al.* (15) also indicated the formation of IBAsp from exogenous applied IBA. Wiesman *et al.* (27) demonstrated that IBA as well as IAA were rapidly metabolized in mung bean and that conjugation is the major pathway of IAA and IBA metabolism in this tissue. The IBA conjugates were identified as IBAsp and at least two high mol wt conjugates, in which IBA is coupled by an amide linkage (28). An IAA-peptide with a similar mol wt was also demonstrated in *Phaseolus vulgaris* (4). Our report deals with the *in vivo* conversion of IAA to IBA and the identification and characterization of the reaction product.

MATERIALS AND METHODS

Plant Material

Seeds of maize, *Zea mays* L. var Hazera 224 and Inrakorn (treated with fungicides), were soaked with water for 2 h and cultivated under sterile conditions in the presence of 0.1% streptomycin sulfate in the dark at 23°C with 90% humidity. The plant material was harvested after 8 d.

Incubation with [^{14}C]IAA and [^{13}C]IAA

Feeding experiments were carried out with either [1-^{14}C] IAA (Amersham, specific activity 1.85 TBq mol^{-1}) or with [$^{13}C_6$]IAA. The appropriate amount (25 ng [^{14}C]IAA; 500 ng [^{13}C]IAA) was added to 5 mL of 100 mM Mes-KOH buffer, pH 6. For the pH dependence studies, the buffer was brought to the appropriate pH, which was monitored during the incubation time. Leaves, roots, and coleoptiles of maize were cut into 2 mm segments and washed in substrate-free buffer. After drying the plant material on filter paper, 1 g of material was added to the incubation solution for various time intervals at 25°C. Control experiments were carried out without tissue in the feeding solution to exclude chemical conversion of IAA to IBA. Several experiments were performed in the presence of 0.1% streptomycin sulfate. After incubation, the tissue was filtered and washed with 50 mL of substrate-free buffer, homogenized with 70% acetone with an Ultra Turrax (20,000 rpm), and centrifuged for 10 min at 5000*g* and the supernatant evaporated to the aqueous phase. The aqueous residue was adjusted to pH 3.0 with 2 N HCl and extracted twice with equal volumes of ethyl acetate. The combined organic phases were dried over Na_2SO_4 and, after concentration, used directly for TLC, HPLC, and/or GC-MS analysis. The uptake of radioactivity in the tissue as well as the total recovery of radioactivity in the acetone phase after extraction were deter-

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[2] Abbreviations: IBA, indole-3-butyric acid; IBAsp; indolebutyryl-aspartic acid; RIC, reconstructed ion chromatogram.

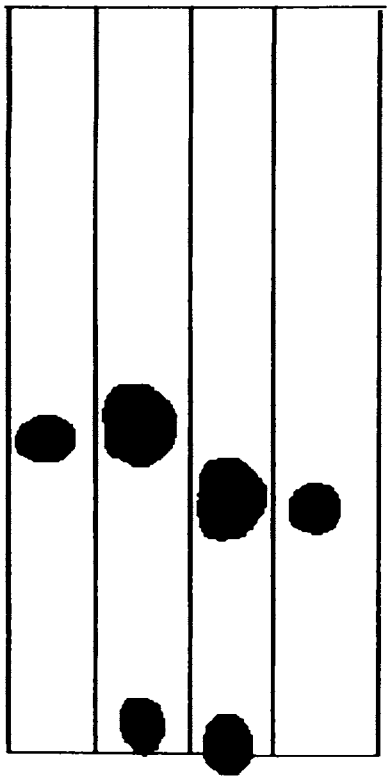

Figure 1. Thin layer chromatograph of methylated samples and of standards of authentic methylated IAA and IBA. The samples used for the TLC analysis were obtained after HPLC of the extract from the corn variety Inrakorn (total seedlings). HPLC conditions were as described in "Materials and Methods." TLC solvent was hexane:ether (60:40, v/v); visualization done with Ehmann reagent.

mined by taking appropriate aliquots and determining their radioactivity (details below).

TLC Analysis

The ethyl acetate fraction was chromatographed on silica gel F_{254} plates (Merck) with chloroform:acetic acid (95:5, v/v) as solvent. Identification was achieved by co-chromatography with authentic standards by fluorescence quenching at 254 nm (R_F values of IAA and IBA were 0.45 and 0.6, respectively). The results were confirmed on silica gel plates with benzene:dioxan (65:35, v/v) as solvent. The chromatogram was then divided horizontally in 0.5 to 1 cm zones and the silica gel was scraped off into scintillation vials. The radioactivity of the different zones was then determined after adding 1 mL methanol and 3 mL of scintillant (Szintillator 199, Packard) in a Packard 2000 CA Tri Carb liquid scintillation counter equipped with an IBM PC/XT computer. Alternatively, the sample was chromatographed on HPLC and the radioactive IAA and IBA fractions were pooled, evaporated to dryness, and methylated with diazomethane (7) prior to TLC analysis on silica gel plates with hexane:ether (60:40, v/v) as solvent. Visualization was done with Ehmann reagent (8).

HPLC Analysis

The methylated IAA and IBA from TLC plates and the ethyl acetate extract were analyzed by HPLC using a reverse-phase column (Biotronik-J., finepak SILC 18-5, Maintal, FRG; pre-column from Guard-PAK, C_{18}, Waters Associates, Milford, MA) and UV detection at 280 nm. Solvents were 55% methanol containing 1% acetic acid for the nonmethylated samples and 60% methanol for the methylated samples. Flow rate was 0.8 mL min^{-1}. Identification was achieved by co-chromatography with authentic standards. Fractions of 0.4 mL were collected and counted as above or used for subsequent GC-MS analysis.

GC-MS Analysis

GC-MS identification was performed with a Finnigan model MAT 4600 using electron impact ionization. The gas chromatograph was equipped with a Durabond-5 column, 30 m × 0.25 mm, 0.25 μm, film (J&W Scientific, Folsom, CA). Temperature program was 140°C for 2 min, following by an increase of 10°C min^{-1} to 200°C and of 20°C min^{-1} to 250°C. Under these conditions, IBA was eluted after 10.5 min. Spec-

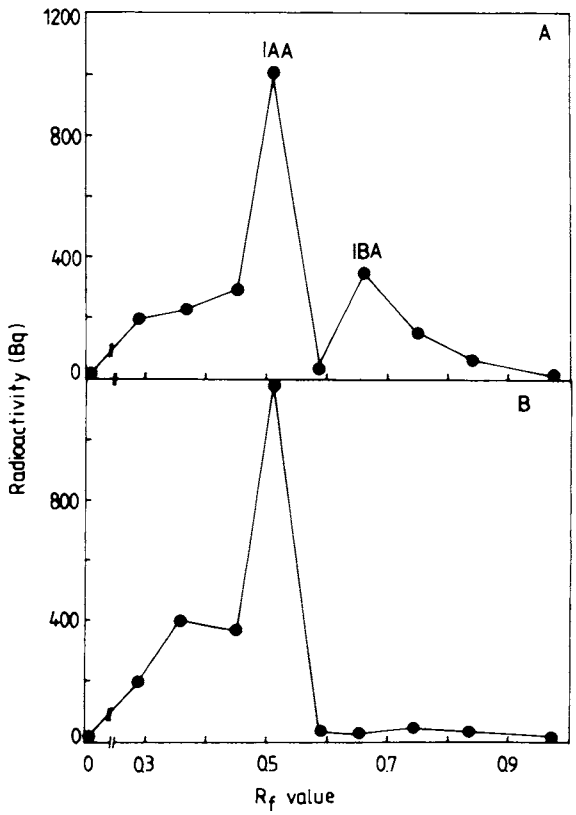

Figure 2. Distribution of [^{14}C] on TLC chromatogram of extract after 1 h incubation of corn leaves (var Inrakorn) with 5 kBq [^{14}C]IAA. Solvent was chloroform:acetic acid (95:5, v/v). A, Sample; B, control without tissue.

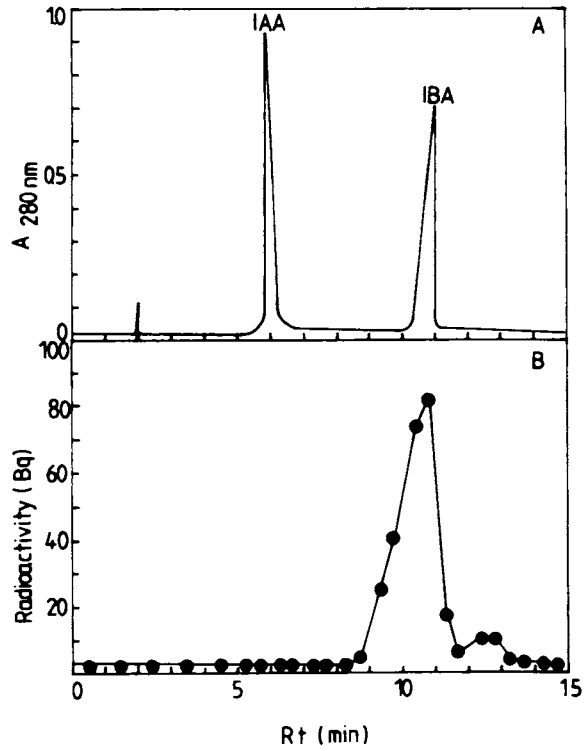

Figure 3. HPLC separation of the IBA fraction from TLC. Solvent was methanol:acetic acid:water (55:1:44, v/v). Flow rate, 0.8 mL min^{-1}. A, Standard chromatogram of IAA and IBA; B, radioactivity profile of the IBA fraction.

tra were taken by both continuous and RIC scans and by selected ion monitoring. The results of the mass spectra were confirmed by library search.

Alkaline Hydrolysis

An aliquot of the plant extract was evaporated to dryness and hydrolyzed with 7 N NaOH under N_2 at 100°C for 3 h. After hydrolysis, the solution was diluted with water to 1 mL and brought to pH 3.0 with HCl. The solution was extracted with ethyl acetate and used for TLC analysis for the determination of free IBA as described above.

Statistical Treatment of the Data and Confirmation of Results

All experiments, except GC-MS analysis, were done three to five times at the Botanical Institute in Frankfurt, Germany. Confirmation of the experiments was provided by repeating the TLC and HPLC analysis in Volcani Center, Israel, with independent cultured plants. All results present means of independent experiments. Mean SE was ≤10%. The K_m values were calculated by linear regression analysis of the data after Lineweaver-Burk transformation.

RESULTS

Identification of the Reaction Product

IBA was identified as an endogenous compound in the corn variety Inrakorn by HPLC followed by TLC (Fig. 1) and GC-MS (see Fig. 4B). All feeding experiments were carried out with both varieties (Hazera 224 and Inrakorn). Because there were no significant differences between the two varieties, representative experiments with only one variety are presented. After feeding of [1-^{14}C]IAA to 2 mm segments of corn shoots and roots, a labeled compound was detected by TLC corresponding to authentic IBA standard (Fig. 2A). The control experiments without tissue showed no significant label at the R_F of IBA (Fig. 2B). No differences were found between the experiments performed with or without streptomycin sulfate (data not shown), indicating that no bacterial activity was involved in the process. The zone corresponding to IBA was eluted from TLC and chromatographed on HPLC. The eluate was collected and the radioactivity determined and compared to a co-chromatogram with authentic IBA standard (Fig. 3A, B). Similarly, the methylated sample co-migrated with authentic methyl IBA standard on TLC (data not shown). [$^{13}C_6$]IBA was identified in the plant extract following incubation with [$^{13}C_6$]IAA (Fig. 4D). Figures 4A, B, and C show the RIC and mass spectra of methylated authentic IBA and methylated samples of the corn varieties Inrakorn and Hazera 224, respectively. Figure 4D shows the reconstructed ion

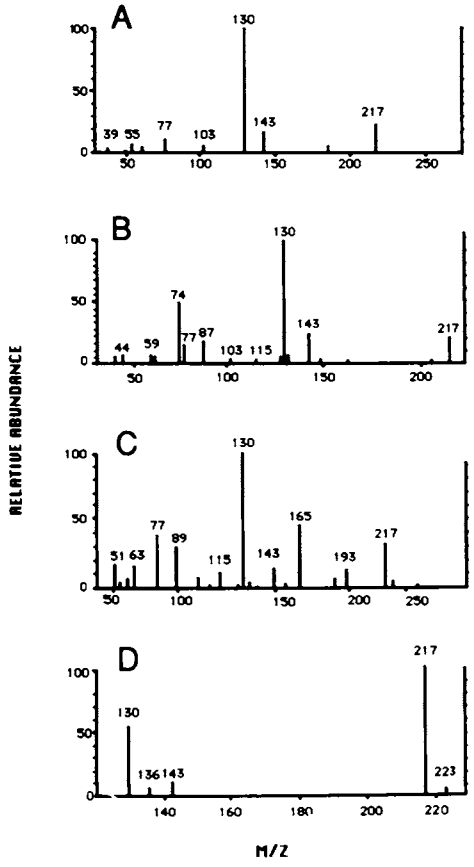

Figure 4. The 70 eV impact mass spectra of (A) methylated IBA standard, (B) methylated endogenous IBA isolated from leaves of *Zea mays* L. var Inrakorn, and (C) endogenous methylated IBA isolated from leaves of *Zea mays* L. var Hazera 224. D, Monitoring ion detector RIC of methyl[$^{13}C_6$]IBA after incubation of leaves of *Zea mays* L. var Hazera 224 with [$^{13}C_6$]IAA.

chromatography for ions 130, 136, 217, and 223 of the reaction product after the *in vivo* experiment. The mass spectrum of the RIC peak at retention time 10.5 min yielded the fragmentation pattern of typical 3-substituted indoles. The molecular ions of *m/z* 217 and 223 are those of [^{12}C]IBA and [^{13}C]IBA, respectively. The cleavage of the side chain with retention of the methylene carbon and ring expansion resulted in a base peak at *m/z* 130 and 136, the quinolinium ions of those molecules.

Characterization of the Reaction

After 8 d, the two maize varieties showed differences in root morphology (Fig. 5). The maize variety Inrakorn (Fig. 5A) possesses longer, more hairy roots, whereas the variety Hazera 224 (Fig. 5B) has little roots with a small number of root hairs under the same culture conditions. When roots, leaves, and coleoptiles of the two varieties were harvested separately and incubated with [^{14}C]IAA, they showed different rates of formation of IBA after 1 h incubation (Table I). Hazera 224 had the highest IBA formation rate in the leaves, with 4.2% of the IAA taken in by the plant, whereas in roots and coleoptiles, only 1.1% and 1.5%, respectively, of total IAA uptake was converted to IBA. Contrary to the IBA formation in Hazera 224, IAA conversion in Inrakorn was nearly the same in both leaves (2.4%) and roots (1.7%), whereas in the coleoptiles, only 0.8% of IAA taken in was converted to IBA. The time course study of IBA formation in roots and leaves of the two varieties showed a maximum after 2 h in the leaves of Hazera 224 and after 1 h in the roots of Hazera 224 and in the leaves of Inrakorn (Fig. 6). In roots of Inrakorn, IBA formation reached maximum after 2 h, but showed no decline up to 4 h incubation. Alkaline hydrolysis of the extracts from Hazera 224 leaves showed 25% more [^{14}C]IBA than without hydrolysis (data not shown). The pH optima for IAA uptake and IBA formation were at 6.0 and 7.0, respectively (Fig. 7). Both maize varieties had similar pH optima for IAA uptake and IBA formation; therefore, data of the experiment with the variety Inrakorn are representative for both varieties. After incubation with different IAA concentrations, IBA formation showed typical Michaelis-Menten kinetics. Following transformation of the data into a Lineweaver-Burk plot (Fig. 8), K_m values of 17 and 25 μM for the IBA formation were determined in leaves and roots of Inrakorn and Hazera 224, respectively.

Table I. *In Vivo Conversion of [^{14}C]IAA to [^{14}C]IBA by Various Tissues of Two Varieties of Zea mays*

One gram fresh weight of roots, leaves, and coleoptiles of *Zea mays* L. var Inrakorn and Hazera 224 were incubated with 5 kBq [^{14}C]IAA for 1 hr at 25°C. TLC (silica gel) with chloroform:acetic acid (95:5, v/v) as solvent.

Variety	Tissue	Uptake of [^{14}C]IAA	[^{14}C]IBA Formed	IBA Conversion
		Bq		%
Hazera 224	Roots	2514	28	1.1
	Coleoptiles	1225	18	1.5
	Leaves	1736	73	4.2
Inrakorn	Roots	1944	32	1.7
	Coleoptiles	1735	14	0.8
	Leaves	1540	36	2.4

DISCUSSION

In the present study, we have shown that roots and shoots of two different corn varieties are able to convert IAA to IBA. Both the ring system (indole moiety) and the side chain of the newly formed IBA were found to derive from IAA. Feeding of [$^{13}C_6$]IAA, in which the ^{13}C atoms are in the benzene ring, resulted in the formation of [^{13}C]IBA (as demonstrated by GC-MS), showing that the labeled ring system is preserved. Feeding with [^{14}C]IAA labeled in the 1-position resulted in radioactively labeled IBA, meaning that the car-

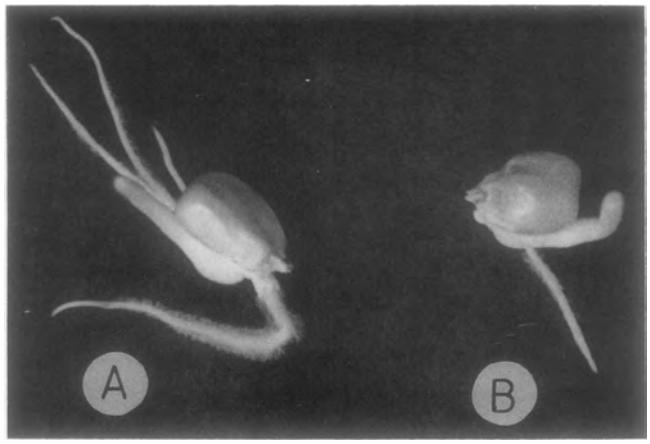

Figure 5. Eight-day-old seedlings of *Zea mays* L. var Inrakorn (A) and var Hazera 224 (B). Note the differences in root hair development between the two varieties.

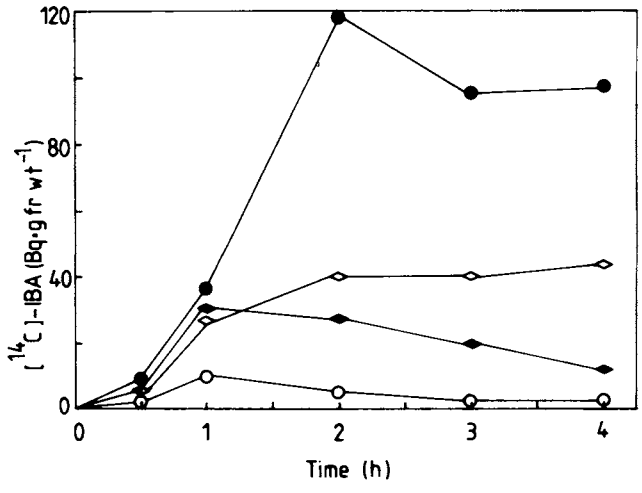

Figure 6. Time course of IBA formation from [^{14}C]IAA over a period of 4 h. (●) *Zea mays* L. var Hazera 224, leaves; (○) *Zea mays* L. var Hazera 224, roots; (◆) *Zea mays* L. var Inrakorn, leaves; (◇) *Zea mays* L. var Inrakorn, roots. All values are corrected for [^{14}C]IAA uptake.

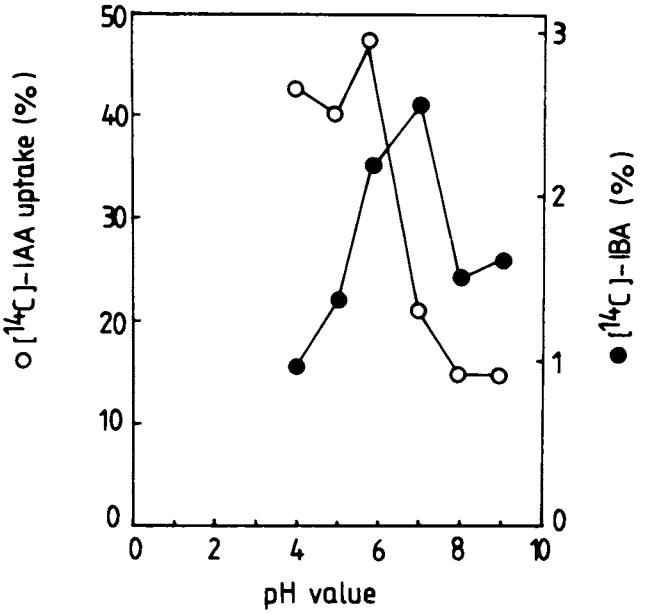

Figure 7. pH dependence of IBA formation from IAA. For every experiment, 1 g leaf tissue of *Zea mays* L. var Inrakorn was incubated for 1 h at 25°C in 100 mM Mes buffer with 5 kBq of [^{14}C]IAA at different pH values. (●) IBA formation; (○) IAA uptake.

boxyl group is maintained in the biosynthesis of IBA from IAA.

It is possible that the conversion of IAA to IBA follows the primary steps of fatty acid biosynthesis, in which acetyl moieties are transferred to an acceptor molecule (IAA) (26). Bandurski and Schulze (personal communication) demonstrated the acetylation of IAA by injecting IAA and acetyl-CoA simultaneously into corn kernels. A head-to-tail acylation of acetyl-CoA by a second molecule of acetyl-CoA resulting in acetoacetyl-CoA would build the butyryl side chain of IBA. Alternatively, two acetyl-CoA molecules are added sequentially to the IAA residue, thus forming the 4-carbon side chain. Patel and Walt (21) investigated the substrate specificity of acetyl-CoA synthetase from yeast. They found that the enzyme was not very specific and that it had some flexibility in accepting a variety of small carboxylic acids (*e.g.* CH_3-CH_2-COOH) as substrates. As there is also evidence for indole-3-propionic acid as a natural compound in plant tissue (24, 25), this might be a reasonable pathway for the biosynthesis of both compounds from IAA. So we can propose a biosynthetic pathway for the biosynthesis of IBA from IAA analogous to the first steps in fatty acid biosynthesis involving an acetyl-CoA synthetase (22), and probably also an acetyl-CoA carboxylase (17) and an acyl-CoA transferase (3) to transfer the acetyl units to IAA.

We have demonstrated that IAA was converted to IBA in the roots, coleoptiles, and leaves of corn seedlings (Table I). Overall, the highest rate of IBA formation was found in the leaves of Hazera 224 (4.2%). Significantly more IBA was found in the roots of the maize variety that formed an extensive root system (Inrakorn) than in the one with the small root system (Hazera 224). We still do not have enough experimental evidence to attach any biological significance to the conversion of IAA to IBA and its relationship to root formation. Many reports deal with the better effect of IBA *versus* IAA on the formation of lateral roots (11, 16, 20), and it was suggested that the higher rooting ability of IBA is due to the higher stability of the former compound to oxidation in the plant (16, 20). However, Wiesman *et al.* (27) were not able to find any significant differences in the rate of metabolism between these two auxins in mung bean cuttings, and there are reports that in some species, IAA even decreases rather than promotes the number of roots formed (9). One possibility is that IBA and IAA form different conjugates in the specific tissues. Epstein and Wiesman (11) demonstrated that IBA-alanine had a better effect on the number of roots induced in olive cuttings compared with free IBA. Wiesman *et al.* (28) showed that both IAA and IBA formed conjugates with aspartate in mung bean cuttings, but IBA also formed another conjugate, probably an IBA peptide.

The time course study of IBA formation in maize leaves showed a decrease of radioactivity in the IBA fraction after 1 and 2 h for the varieties Inrakorn and Hazera 224, respectively (Fig. 6). This decline might be due to conjugation of IBA during the incubation time. Higher rates of free IBA were only detected after alkaline hydrolysis with 7 N NaOH at high temperature, which cleaves all conjugated forms, but not after alkaline hydrolysis with 1 N NaOH at room temperature (J. Ludwig-Müller, unpublished results), at which auxins are only released from ester conjugates (5). These data, as well as the other data mentioned above, might prove that amide conjugates of IBA are important as a source of free auxin and, therefore, have a regulatory function in the rooting process.

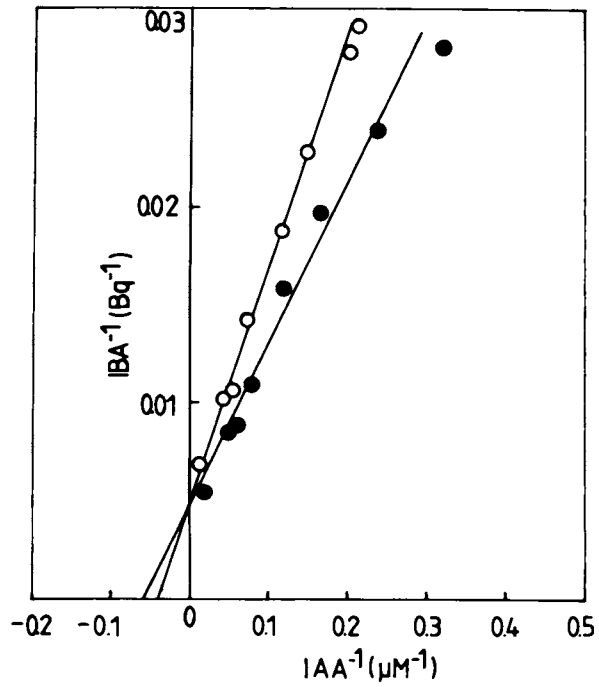

Figure 8. Lineweaver-Burk plot of substrate kinetics. (●) leaves of *Zea mays* L. var Inrakorn; (○) leaves of *Zea mays* L. var Hazera 224. The K_m values were determined as 25 and 17 µM, respectively.

However, further experiments should be carried out on this subject to generate more information about the role of these substances in the plant.

The conversion rate of IAA to IBA (2–4% of total radioactivity uptake, Table I) is in agreement with other conversion rates determined for *in vivo* reactions in the biosynthesis of IAA. Ludwig-Müller and Hilgenberg (19) found a formation rate for individual indole derivatives of 4 to 13% of total radioactivity uptake after feeding of *N*-DL-malonyltryptophan to segments of Chinese cabbage. Helmlinger *et al.* (18) found rates of 8 to 15% for the conversion of indole-3-acetaldoxime to indole-3-acetonitrile in Chinese cabbage, while Rausch *et al.* (23) determined rates of approximately 3% in benzene-soluble products and approximately 12% in water-soluble products after feeding of tryptophan to segments of *Brassica napus*. The low K_m value for the conversion of IAA to IBA, indicating high affinity for the substrate IAA (Fig. 8), allows even small amounts of IAA (*i.e.* only parts of total IAA present in plant tissues) to be converted to IBA.

ACKNOWLEDGMENTS

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Indole-3-butyric acid (IBA) production in culture medium by wild strain *Azospirillum brasilense*

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Abstract

Some microorganisms found in the soil are able to produce substances which regulate plant growth. In this study, we show the presence of a substance associated with auxin activity, identified as indole-3-butyric acid (IBA), in *Azospirillum brasilense* UAP 154 growth medium. *A. brasilense* was grown and indolic compounds were extracted from the supernatant. These were then analyzed by high performance liquid chromatography (HPLC), gas chromatography and gas chromatography mass spectrometry. The retention time was similar to those of the authentic IBA standard. The compound obtained from HPLC was collected and applied to maize seedlings (*Zea mays*), inducing biological activity along the roots, similar to that induced by an authentic IBA standard.

Keywords: Indole butyric acid; Indole-3-butyric acid; High performance liquid chromatography; Phytohormone; *Azospirillum brasilense*

1. Introduction

Plant growth regulators (phytohormones) are organic substances, which at low concentrations (less than 1 mM), promote, inhibit, or modify the growth and development of plants. Commonly six major groups of phytohormones are recognized: gibberellins, cytokinins, abscisic acid, ethylene, brassinosteroids and auxins [1,2]. The plant hormone (auxin, indole-3-acetic acid (IAA)), is an organic compound synthesized in one part of the plant and translocated to another, where in very low concentrations it causes a physiological response as a curvature of oat coleoptiles towards light [2].

Among auxin-like compounds, there exist indole-3-propionic acid (IPA), indole butyric acid and naphthalen acetic acid. Some of these, such as indole-3-butyric acid (IBA) occur naturally in more plants [1].

Blommaert [3] found greater amounts of IBA than of IAA at sprouting onset. Likewise, Bayer [4] also found that *Nicotiana* tumors have more IBA than normal tissues. IBA has also been identified in peas, maize and carrot tissues inoculated with *Agrobacterium rhizogenes* [4].

One of the factors that dramatically limits grain production is the capacity of plants to take in nitrogen from the soil. The need to increase grain production has been achieved with the addition of nitrogen fertilizers, among other agricultural practices; however, when used in excess these are harmful to world ecology, and besides their manufacture is quite expensive. A substitute for chemist nitrogen fertilizers is biofertilization with certain bacteria, cyanobacteria and actinomycetes, that when applied to plants, may provide beneficial effects through the supply of fixed nitrogen and the production of phytohormones [5]. One of these nitrogen-fixing bacteria is *Azospirillum* spp., which has been isolated from grain plants, forage grass, and cacti [6–8]. The *Azospirillum* genus appears in certain species;

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Abbreviations: Trp, tryptophan; IAA, indole-3-acetic acid; IPyA, indole-3-pyruvic acid; TAM, tryptamine; TOL, indole-3-ethanol; IBA, indole-3-butyric acid; IPA, indole-3-propionic acid; IAm, indole acetamide; ILA, indole lactic acid; BSTFA, bistrimethilsililfluoroacetamide; TMCS, trimethilchlorosilan

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Azospirillum amazonense [9], *Azospirillum brasilense* [10], *Azospirillum dobereinerae* [11], *Azospirillum halopreferens* [12], *Azospirillum irakense* [13], *Azospirillum largimobile* [14], *Azospirillum lipoferum* [10] and others (http://www.ncbi.nlm.nih.gov/Taxonomy). *Azospirillum* has great potential as a growth phytostimulator. Inoculation of grain crops of agronomical importance with these bacteria causes an increase in grain production [15].

Besides fixing nitrogen, *Azospirillum* produces auxins, such as IAA which cause an increase in root hair production [16], thus improving nutrient uptake from the soil [17]. Another auxin associated with *A. brasilense* inoculation is IBA, which has been found in the roots of maize seedlings inoculated with this microorganism [18].

Several reports indicate the existence of microorganisms associated with plant roots, or colonizing internal tissues in which the production of plant growth-regulating substances has been shown. Auxins have been identified in *Azotobacter chroococum* and *Azotobacter vinelandii* [19], in *Rhizobium* spp. [20], *Agrobacterium tumefaciens* [21,22], *A. rhizogenes* [23], *Bradyrhizodium* spp. [24], *Azospirillum* spp. [16,25], *Acetobacter diazotrophicus* [26,27].

Although IBA has been detected in the root tissues of maize inoculated with *A. brasilense*, it has never been found in the supernatant of a culture grown from this microorganism [18].

Previous studies in our laboratory have determined several intermediates participating in the IAA biosynthetic pathways, in wild and mutant strains of *A. brasilense*. When an IBA standard was introduced into a thin layer chromatography (TLC) assay, where a cell-free culture of *A. brasilense* had been run, the presence of a very weak signal of IBA was identified (unpublished data).

In this study we identified IBA in the wild strain *A. brasilense* UAP 154 by high performance liquid chromatography (HPLC), gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS); furthermore, its biological activity was assayed on maize plantlets.

2. Materials and methods

2.1. Bacterial strains

Bacterial strains used in this work are listed in Table 1.

Table 1
Strains

Strains	Description	Reference
E. coli C600	Sm^r supE hsdR	[39]
A. brasilense UAP 154	Ap^r	[40]
	Rif^r	Velázquez, M., personal communication

2.2. Media and growth conditions

A. brasilense was grown at 32°C on Jain and Patriquin medium [17] supplemented with tryptophan (Trp) and antibiotics Rif or Amp, during 72 h for indolic compounds production. As a negative control, *Escherichia coli* was grown at 37°C in Luria–Bertani (LB) medium and supplemented with antibiotic when required.

2.3. TLC

Wild strain *A. brasilense* UAP 154 was grown at 32°C in a 250-ml Erlenmeyer flask on a rotary shaker at 160 rpm for 72 h. The flask contained 60 ml of Jain and Patriquin medium [17] (supplemented with 100 μg ml^{-1} Trp). The cell-free culture supernatant was adjusted to pH = 2.7 with HCl 1 N and extracted with 1 volume of ethyl acetate at room temperature, vacuum dried at 37°C, and dissolved in 2 ml of methanol. The assay was run in 2D TLC, by using a mixture of eluents: acetone:chloroform:acetic acid (96%) (v:v:v) in the first dimension and chloroform:acetic acid (96%) (v:v) in the second dimension, and then developed with the Salkowski reagent.

2.4. IBA detection by HPLC

Analytical assay: extracted samples were dissolved in methanol and analyzed by HPLC (Ultrasphere ODS-Beckman, programmable solvent module 125 with diode array detector module 168), and eluted with 1% water-acetic acid/methanol gradient for 32 min in the following program: at the moment of initiation, 60% acetic acid and 40% methanol for 13 min; at 13.01 min, 0% acetic acid and 100% methanol for 10 min; at 23.01 min, 60% acetic acid and 40% methanol for 9 min. Detection was performed at 280 nm.

Preparative assay method for IBA isolation: in order to increase the amount of IBA from wild strain, an analysis by HPLC in preparative column (Ultrasphere ODS-Beckman) was performed. Conditions were as those described above and samples were taken after column fractioning purification every 2 min, and subsequently each fraction was assayed in analytical column under the same conditions.

2.5. GC

The material obtained and dissolved in methanol was derived with bistrimethilsililfluoroacetamide (BSTFA) and trimethilchlorosilan (TMCS). The derivation reaction was as follows: 75 μl of pure indolic compounds and that extracted from the methanol (obtained previously), were completely evaporated, dissolved in 30 μl of pyridine and 45 μl of BSTFA and then 1% of TMCS was added. The mixture was incubated at 60°C in a double boiler for 2 h.

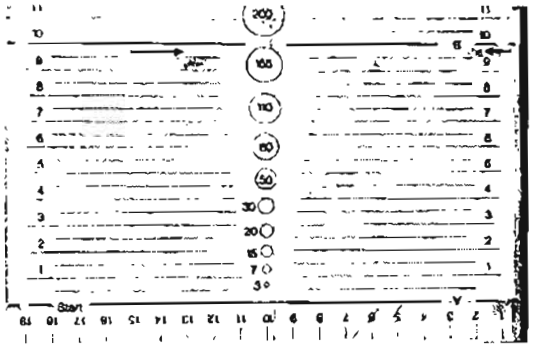

Fig. 1. 2D TLC of a cell-free supernatant of wild strain *A. brasilense* UAP 154 and authentic tryptamine (TAM), IAN, indole-3-pyruvic acid (IPyA), ILA, indole acetamide (IAm), IAA, indole-3-ethanol (TOL) and IBA as standards. The arrows refer to the presence of IBA. In sample 2, IBA, 3, TOL; 4, IAm; 5, ILA; 6, IPyA; 7, TAM (R_f 4.5)/IAA (R_f 9); 8, Trp (R_f 4)/IAN (R_f 9.5); 13, UAP 154.

From each sample, 1 μl was injected into a silica cast capillary column (J&W Scientific DB-17 of 30 m×0.55 mm of i.d. and 1.0 mm of film thickness, Varian star 3400 CX). The running parameters were: 2 min at 173°C, followed by increments of 10°C min^{-1} in the column temperature until it reached 250°C, which was maintained for a further 3 min. The injector was adjusted to 250°C and the carrier gas flow (N_2) was 10 ml min^{-1} [28–30].

2.6. GC-MS

With the aim of obtaining a more accurate identification of IBA from *A. brasilense* UAP 154, GC-MS was performed. From samples obtained by HPLC, 1 μl was injected in the split-splitless mode in a GC-MS system (HP 5890 Series II GC), with operation and data analysis, using Chemstation software under the following conditions: column: link cross methylsilicone, 12 m×0.25 mm of o.d. method: temperature 40–270°C, flux 1 ml min^{-1}, split 1:25, scale 15–350.

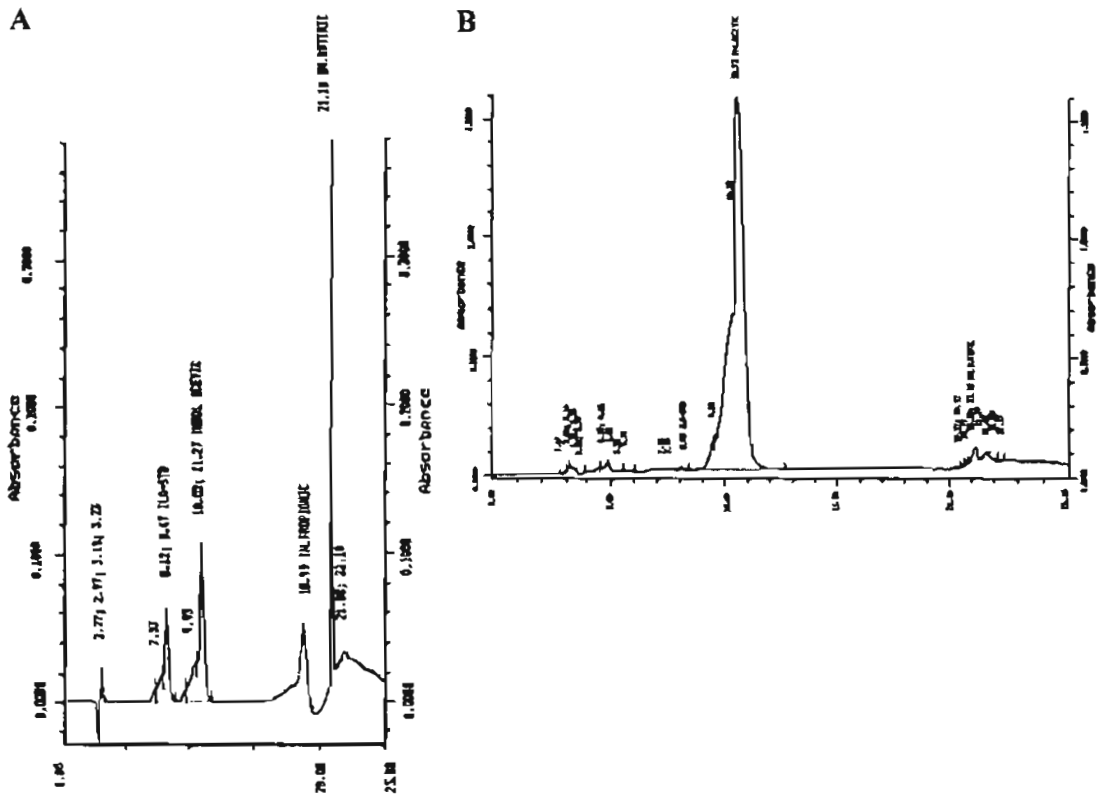

Fig. 2. Reverse-phase HPLC profiles. A: Authentic indolic compound standards. Retention times: ILA, 8.47; IAA, 10.82; IPyA, 18.99; and IBA, 21.18 min. B: Indolic compounds produced by wild strain *A. brasilense* UAP 154, retention time IBA is 21.19 min, culture in minimal medium with 100 μg ml^{-1} Trp added. The column was eluted in mobile phase methanol–acetic acid 1%.

2.7. Biological assay

Creole maize (*Zea mays*) seeds were surface sterilized with chloramine T (10%) for 10 min, then vigorously washed three times with sterile water, and subsequently germinated on Petri dishes lined with filter paper and kept in a humid chamber in the dark. 24 h after germination, the seedlings were put into a hydroponic system and the standard and purified IBA (12 μM) were applied. Controls consisted in the addition of culture medium (15 μl) and methanol (15 μl) into the hydroponic solution. Longitudinal growth of the root and root hair production was measured after 72 h. After that, the mean root dry weight was determined in tree plants, for each treatment four times over.

3. Results

3.1. Detection of IBA in A. brasilense

The first evidence for the presence of IBA in the supernatant of *A. brasilense* was obtained using 2D TLC where a weak signal co-migrated with the standard IBA (Fig. 1).

3.2. Detection of indolic compounds by HPLC in A. brasilense

The detection of standard indolic compounds showed retention times as follows: indole lactic acid (ILA), 8.47 min; indole acetic acid, 10.82 min; indole propionic acid, 18.99 min; and indole butyric acid, 21.18 min (Fig. 2A).

Indolic compounds related production from *A. brasilense*.

Fig. 2B shows the HPLC chromatogram of the wild strain *A. brasilense* as UAP 154 (IBA 1.158 μg ml^{-1} growth medium, S.D. = 0.006149, S.E.M. = 0.003550) where a peak appears with a retention time of 21.18 min similar to the one presented by the authentic IBA standard (Fig. 2A). Another indolic compound with retention time of 10.82 min was the IAA (59 μg ml^{-1} growth medium, S.D. = 1.143, S.E.M. = 0.8083).

Fig. 2A shows the HPLC chromatogram of authentic indolic compound standards. Together with the production of some indolic compounds in *A. brasilense* the presence of IBA could be assessed with the corresponding standards.

3.3. Detection of IBA from A. brasilense by GC

With the aim of detecting IBA by GC, samples were derived (as described in Section 2) and subjected to chromatography. Fig. 3A shows GC chromatogram with retention times of the authentic indolic compound standards: retention time of IBA: 14.5 min; of IPA: 10.6 min; of IAA: 9.85 min.

Fig. 3B shows a GC chromatogram of wild strain *A. brasilense* UAP 154 (IBA 1.158 μg ml^{-1} growth medium)

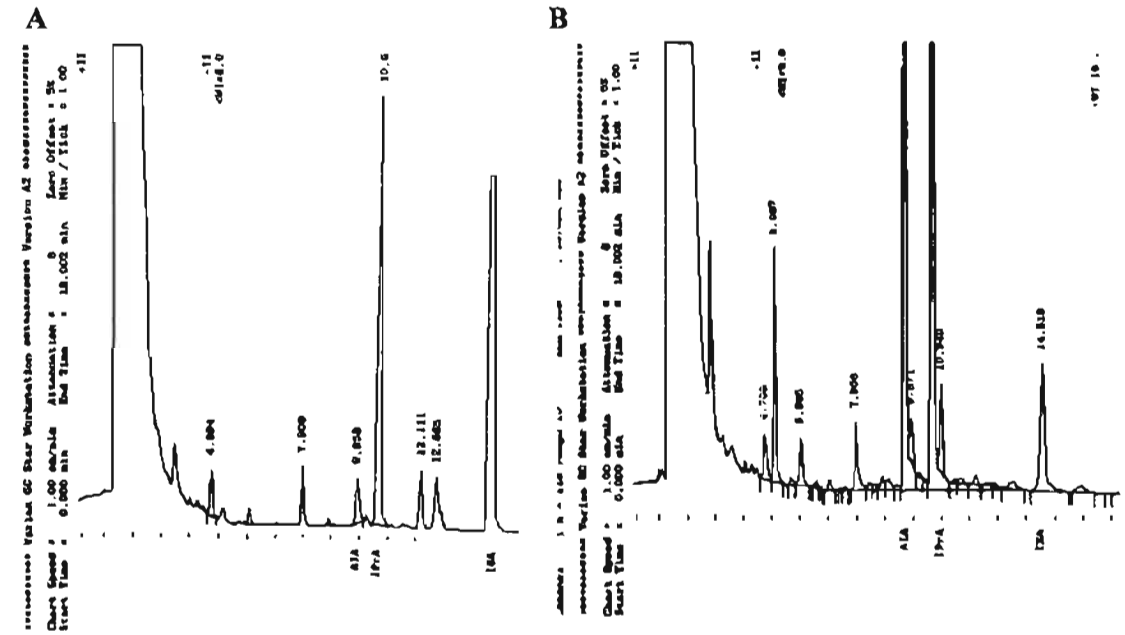

Fig. 3. GC profiles. A. Authentic IAA, IPyA and IBA standards, the retention times were IAA, 9.85 min; IPyA, 10.6 min; and IBA, 14.4 min. B: Indolic compounds produced by wild strain *A. brasilense* UAP 154, the retention time was 14.5 min.

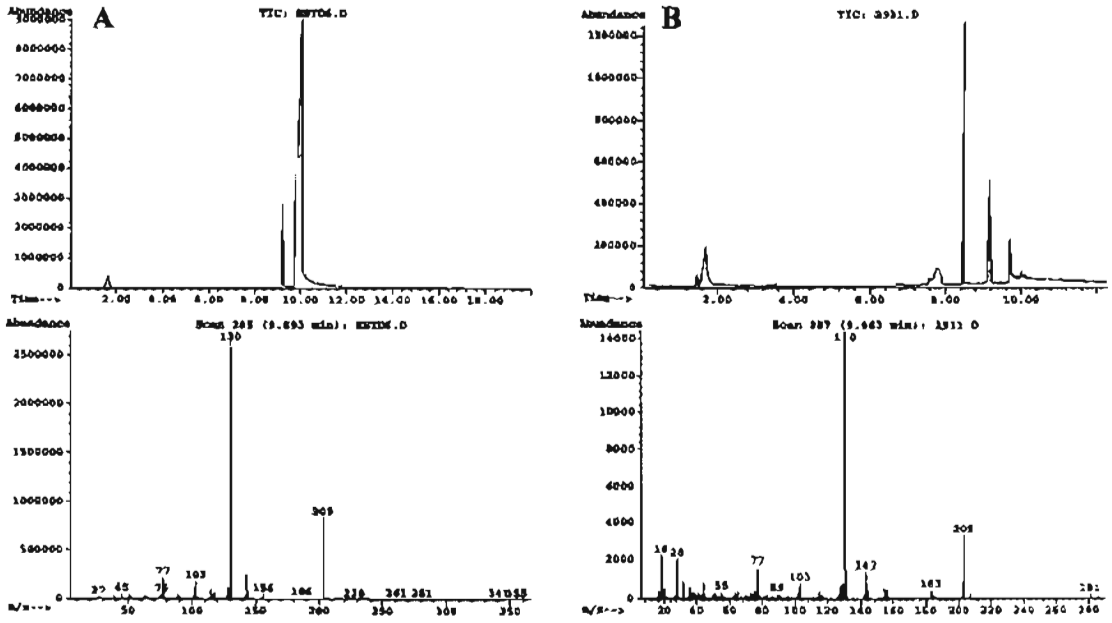

Fig. 4. GC-MS analysis of ion profiles of authentic IBA standard, retention time is 9.71 min (A) and IBA produced by wild strain *A. brasilense* UAP 154 (B) extracted from the acidified culture supernatant cell-free incubated 72 h in minimal medium with 100 μg ml^{-1} Trp added.

with the appearance of a peak with a retention time (14.5 min) similar to the one presented by the authentic IBA standard (Fig. 3A). IAA was present with retention time similar to IAA standard.

3.4. Detection of IBA in A. brasilense by GC-MS

Fig. 4 shows the comparison between two GC-MS chromatograms: the IBA standard control (Fig. 4A) and the IBA produced by wild strain *A. brasilense* UAP 154 (Fig. 4B) (IBA 1.158 μg ml^{-1} growth medium), with a retention time similar to the authentic IBA standard retention time 9.71 min (Fig. 4A). The presence of characteristic ions of IBA was similar in both cases.

3.5. IBA biological assay

Plant biological assays showed that IBA (12 μM) produced by *A. brasilense* acts as an auxin in maize (*Z. mays*), promoting lateral root formation and inhibiting root elongation (Fig. 5). The root dry weight with the addition of IBA standard was 0.0379 g (S.D. = 0.014349, S.E.M. = 0.007175). IBA produced by *A. brasilense* was 0.0658 g (S.D. = 0.011045, S.E.M. = 0.005523). medium growth was 0.0483 g (S.D. = 0.017115, S.E.M. = 0.008558). IAA standard and methanol data are not shown.

The present observations on bioassays in maize seedlings bound to proofs in chromatography prove the existence of IBA in the supernatant of the *A. brasilense* culture.

4. Discussion

It is known that many nitrogen-fixing bacteria are often referred to as plant growth-promoting rhizobacteria (PGPR) because of their capacity to produce plant growth

Fig. 5. Effect of authentic IAA (A) and IBA (B) standards and IBA produced by *A. brasilense* UAP 154 wild strain (C), on adventitious root formation of *Z. mays* shoots grown in vitro. Treated plants were incubated in photo-period light/dark 14/10 h for 3 days at 28°C on media containing 12 μM of each indolic compound. Control shoots were incubated on medium (D), and medium containing methanol (E).

regulators such as indole acetic acid and gibberellins, among other features.

Although IBA is an auxin produced synthetically for application as a rooting agent, Blommaert [3] indicated that it has been identified as a natural product of potato skin, using paper chromatography and bioassays. In this work we report the presence of IBA in the supernatant of *A. brasilense* UAP 154 growth culture by TLC, HPLC, GC, GC-MS and bioassay in maize seedlings. Because of this, when referring to *A. brasilense* as a PGPR bacteria, the IBA production should be mentioned.

The linear gradient conditions used by Alvarez [31] in HPLC determination assays for IAA, were similar to those presented in this work for IAA and IBA. In his work, Alvarez shows the presence of these compounds to be present in apple shoots cultured in vitro through HPLC analysis [31], but does not develop this further with a more complete physical-chemical study.

The IBA from wild strain *A. brasilense* UAP 154 used in this work for GC, GC-MS and biological assays, was obtained from HPLC using acetic acid 1%/water (v/v) and methanol as eluent. Fractions were collected at 2-min intervals in the preparative column. These conditions were chosen in order to permit the obtaining of a better chromatogram resolution, showing clear differences between indolic compounds and permitting that more IBA be obtained for subsequent assays. The differences found in Alvarez's method are: the use of acetonitrile/water gradient, collected at 1-min intervals [31].

We have some *A. brasilense* UAP 154 transconjugants with Tn5 inserted, that were analyzed using HPLC. Some transconjugants displayed a variant, producing IAA and IBA simultaneously: IBA overproducing and IAA underproducing or vice versa, suggesting a probable genetic regulation of these indolic compounds (data not shown).

In GC-MS assays, our conditions were similar to those showed by Bastian et al. [32] to detect A_3 gibberellin (GA_3) in *A. diazotrophicus* and *Herbaspirillum seropedicae* cultures, but with some differences: we did not use isotopes, the cells were not sonicated, we only took the supernatant, and we obtained 59 μg ml^{-1} IAA when *A. brasilense* was grown in minimal medium with Trp added, versus 32 ng ml^{-1} IAA from *A. diazotrophicus* in LGIP medium, with 10% of sucrose added, and 7 ng ml^{-1} IAA from *H. seropedicae* in NFb medium. This indicates a less complicated approach for analyzing the production of auxins by GC-MS.

Auxin IBA is very effective in the promotion of adventitious root formation, as demonstrated in apple plants [31]. It was shown that IBA is more effective than IAA when inducing the formation of adventitious roots [33,34], possibly due to the following considerations: (i) IBA seems to be less susceptible to enzymatic degradation than IAA; (ii) IBA can slowly be transformed into IAA, thus providing a secure IAA supplement; (iii) studies by Nordström et al. [35] have shown that the great effectiveness of IBA could be due to its extensive presence in plant tissues; and (iv) IBA can induce tissue-specific responses due to differential sensitivity among cell types.

The IBA biological activity observed in this work on maize plants showed promotion of lateral root formation and inhibition of root elongation (Fig. 5). Similar data were shown by Zolman et al. [36] for *Arabidopsis thaliana* with IBA standard. Furthermore, the beneficial biological activity of IBA on maize plantlets was supported by the root dry weight values obtained, being higher when adding IBA produced by wild strain *A. brasilense* UAP 154 (0.0658 g), as compared with IBA standard (0.0379 g) and the controls. Bhalerao et al. [37] showed de novo IAA synthesis in *Arabidopsis* seedling, being low at early seedling development (4%) and increasing through time (e.g. 37%, 10–11 days after germination). When considering this, it must be remembered that our assays were carried out during the first 4 days after germination with the purpose of avoiding IAA and IBA endogenous activity. IBA production under the natural conditions of plant–bacterium association is perhaps caused by the presence of Trp in root exudates [19].

The production of IBA and other plant growth regulators by *A. brasilense*, together with the production of major outer membrane proteins [38], are two processes that can help in the understanding of the positive effects observed when there is plant–microbe association.

The findings of this study provide significant evidence that IBA was produced by *A. brasilense* and that it causes clear biological activity in root plants.

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Update on Hormones

Rethinking Auxin Biosynthesis and Metabolism

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Charles Darwin cited the 1871 Ph.D. thesis of Theophil Ciesielski when he postulated in 1880 that a "transmitted influence" present in the tip of plant shoots was responsible for gravitropism. Both Darwin and Ciesielski had realized that the influence was affecting growth differentially. This influence was given the name auxin more than 50 years later and subsequently any compound that promoted growth in specific bioassays was defined as an auxin. The chemical structure of the primary plant auxin, IAA (Fig. 1), has been known since the 1930s to be a 3-substituted indole like Trp. Since that time, the prevailing theory has been that IAA is derived from Trp. However, due to lack of convincing evidence, the biosynthetic pathway for IAA in plants is still undefined. Within the last 10 years, development of precise quantitative methods, good model systems for in vivo analysis, and mutants altered in IAA metabolism have resulted in substantial progress in our understanding of IAA biosynthesis. This review focuses on the new and more complex picture of IAA biosynthesis that has emerged as a result of recent experiments.

THE TROUBLE WITH TRP

In the late 1940s and 1950s, studies showed that labeled and unlabeled Trp applied to various plant tissues was converted to IAA. The ensuing biochemical analysis of over 20 different species of plants led to the conclusion that IAA is derived from Trp, albeit through several possible pathways (reviewed by Nonhebel et al., 1993). Predicted intermediates have been shown to incorporate label from Trp and to be present as native compounds in plants. Enzyme activities that catalyze the interconversion of specific intermediates have been identified, and in some cases the genes encoding these enzyme activities have been cloned (De Luca et al., 1989; Bartel and Fink, 1994; Bartling et al., 1994).

Biochemical studies carried out with tissue segments or plant extracts disrupt compartmentalization; therefore, the enzymes that can catalyze the interconversion of Trp to IAA in vitro may, in fact, never come into contact with the required intermediates in intact cells. So, although many studies have demonstrated the competence of plants to convert Trp to IAA, the physiological relevance of the hypothetical pathways remains questionable. Over the past four decades, the assertion that Trp is the precursor to IAA has been questioned several times. Trp is present in vast excess to IAA, thus the incorporation of label from Trp into IAA amounts to only a meager few percent. In early experiments, minimal attempts were made to quantify the total amount of IAA produced, thereby making it difficult to determine if the low level of conversion was physiologically meaningful. Furthermore, many of the experiments were not carried out under aseptic conditions. Since it has been well established that microbes convert Trp to IAA, the argument has been made that bacteria associated with the plants were actually responsible for the observed conversion of Trp to IAA. Perhaps most troubling for the interpretation of these studies is the observation that Trp is readily converted to IAA nonenzymatically upon routine handling in the laboratory. Although these challenges to the prevailing theory of IAA biosynthesis have been noted periodically over the last 40 years, they have been inexplicably dismissed and the hypothesis that Trp is the primary precursor to IAA has persisted.

STABLE ISOTOPE DILUTION GC-MS SETS A NEW STANDARD FOR ANALYZING IAA BIOSYNTHESIS

A critical component for the study of IAA biosynthesis is the ability to accurately quantify the precursors, intermediates, and end products involved. IAA is present in very low abundance and requires more sensitive detection methods than those used for the study of a major metabolic pathway. Isotope dilution analysis coupled with MS has a long history of solving difficult analytical problems in biology and biochemistry. Stable isotope dilution analysis is based on the principle that a heavy-labeled compound (e.g. ^{2}H-, ^{15}N-, or ^{13}C-labeled compounds) of known amount added to a plant extract will behave analogously to the unlabeled endogenous compound throughout the isolation procedure. Since the amount of labeled compound is known, determining the ratio of labeled to unlabeled compound with GC-MS reveals the amount of endogenous, unlabeled compound. Furthermore, the added specificity obtained by monitoring individual ions lessens the emphasis on purity and yield during isolation of the compound to be measured. The application of this technique to IAA

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Abbreviations: GST, glutathione-*S*-transferase; IAN, indole acetonitrile; IBA, indole-3-butyric acid; TDC, tryptophan decarboxylase.

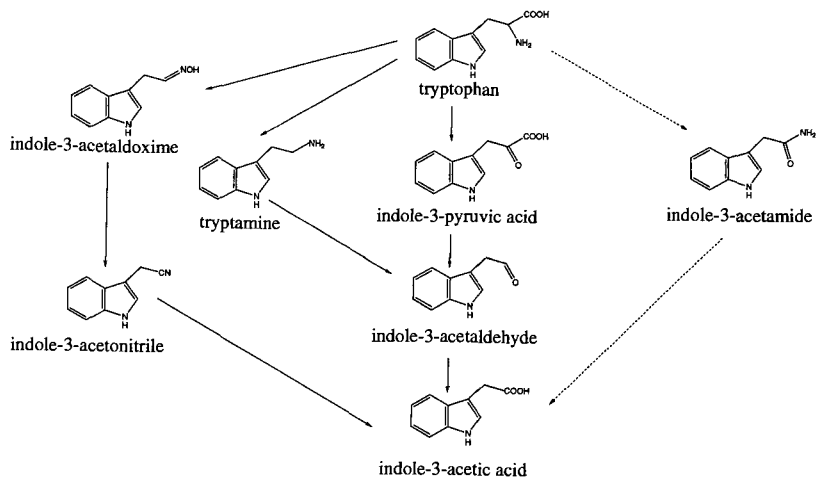

Figure 1. Proposed routes of IAA biosynthesis from Trp. The pathway utilized by microbes is indicated by dashed arrows. Reviewed by Nonhebel et al. (1993).

biosynthesis has increased in the last decade as a result of two important advances. The first was the availability of a reliable internal standard. IAA with ^{13}C substituted at all six of the benzene ring carbons is stable, can be readily distinguished from endogenous IAA by MS, and behaves like the endogenous IAA during purification. Second, significant improvement has been made in the speed with which one can isolate IAA from small amounts of tissue. Good yields can be obtained from as little as 100 mg of tissue in less than 1 d (see Wright et al., 1991; Normanly et al., 1993, and refs. therein). As a consequence, the measurement of IAA levels (and for that matter intermediates in the pathway of IAA synthesis) is now feasible as a routine laboratory procedure.

Stable isotopes are also useful for establishing a precursor-to-product relationship between any two compounds that will incorporate isotope as a result of de novo synthesis. For example, in the case of IAA biosynthesis, if Trp were the sole precursor to de novo-synthesized IAA, then the percent incorporation of isotope into Trp from a labeled Trp precursor (e.g. [^{15}N]anthranilate; see Fig. 2) would be expected to be greater than or equal to the percent incorporation of isotope into IAA. If the percent incorporation of isotope into IAA is greater than that of Trp, there has to be another precursor available to synthesize IAA, in a manner that bypasses Trp, to account for the higher level of isotopic enrichment. This approach provides a means to assess the significance of any compound postulated to be a precursor to IAA.

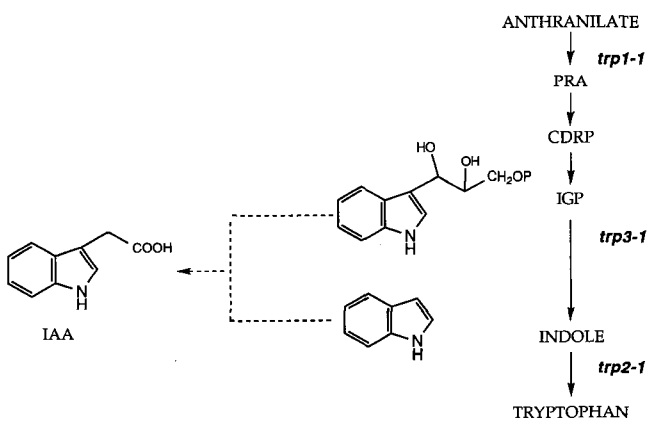

Figure 2. Trp biosynthetic pathway. PRA, *N*-Phosphoribosylanthranilate; CDRP, 1-(*O*-carboxyphenylamino)-1-deoxyribulose phosphate; IGP, indole-3-glycerol phosphate; trp, Trp.

QUANTITATIVE IN VIVO LABELING STUDIES PROVIDE INSIGHT

Aseptic cultures of the aquatic monocot *Lemna gibba* (duckweed) are ideal for stable isotope labeling studies because the entire underside of the plant readily takes up labeled compounds from the surrounding media. Using *Lemna*, the experiments by Baldi et al. (1991) were the first to quantitatively address the question of whether Trp is the precursor to IAA in vivo in intact, growing plants. Their results completely contradicted the then-prevailing theories about IAA biosynthesis (Baldi et al., 1991). *Lemna* fed [^{15}N]Trp, to the extent that 98% of the Trp pool was labeled, incorporated very little ^{15}N into IAA over a period of 5 d. Feeding *Lemna* unlabeled Trp in vast excess had no effect on IAA levels either.

Deuterium oxide, or heavy water, is an excellent compound for labeling studies, since water is generally freely accessible to all compartments of the plant. Deuterium exchange will occur early in the shikimate pathway prior to the synthesis of anthranilate; therefore, knowledge of the precursors and intermediates in IAA biosynthesis is not required. This technique has played a critical role in a number of recent studies on de novo IAA biosynthesis (Baldi et al., 1991; Cooney and Nonhebel, 1991; Wright et al., 1991; Bandurski et al., 1992). In one such study, dark-grown maize seedlings fed deuterium oxide incorporated deuterium into Trp but not into IAA over a 7-d period. This result indicates that synthesis of the two compounds is separable over time (Bandurski et al., 1992).

MUTANTS REVEAL A TRP-INDEPENDENT IAA BIOSYNTHETIC PATHWAY

Because of a lack of auxin biosynthetic mutants, a genetic approach to the problem of IAA biosynthesis has not been

feasible in the past. Although such mutants still have not been identified, analysis of recently identified Trp auxotrophs resulted in some surprising new data that have dramatically changed our view of IAA biosynthesis. Maize and *Arabidopsis thaliana* Trp mutants clearly showed that IAA biosynthesis can proceed without Trp and that a previously unknown IAA biosynthetic pathway likely branches from the Trp biosynthetic pathway at indole or indole-glycerol phosphate (Fig. 2).

Orange pericarp maize is a double mutant defective in both Trp synthase β genes. In vivo labeling with [^{15}N]anthranilate revealed that this mutant does not synthesize Trp ([^{15}N]Trp was not detected) but does synthesize IAA to levels 50-fold above those of wild type. Feeding deuterium-labeled Trp to the mutant did not result in any incorporation of deuterium into IAA, providing strong evidence for a Trp-independent IAA biosynthetic pathway (Wright et al., 1991).

The *trp2* mutant of *Arabidopsis* is defective in one of two Trp synthase β genes and is a Trp auxotroph in conditions of high light intensity. The *trp3* mutant is defective in Trp synthase α and the *trp1-1* mutant is defective in anthranilate phosphoribosyl transferase activity (Last, 1993). Like the *orange pericarp* mutant, both *trp2* and *trp3* mutants accumulate IAA (38- and 19-fold above wild-type levels, respectively) (Normanly et al., 1993). The *trp2* mutant also accumulates indole. Double labeling of the *trp2* mutant with [^{15}N]anthranilate and deuterated Trp revealed that significantly more ^{15}N was incorporated into IAA than into Trp, which argues against Trp being a precursor. The amount of deuterium incorporated into IAA from deuterated Trp was not significantly above the background nonenzymatic conversion of Trp to IAA.

Although in vivo labeling experiments with *Lemna*, maize, and *Arabidopsis* have uncovered IAA biosynthetic pathways that were not detected by previous methodologies, Trp as a precursor to IAA is certainly not eliminated by these results. *Phaseolus vulgaris* seedlings have proven to be another good model system with which to study IAA biosynthesis. In vivo labeling studies of bean seedlings clearly demonstrated that essentially all of the IAA is derived from Trp (Bialek et al., 1992).

MULTIPLE, DEVELOPMENTALLY REGULATED IAA BIOSYNTHETIC PATHWAYS

Careful investigation into the role played by IAA biosynthesis in somatic embryogenesis has yielded very intriguing information about IAA biosynthesis. Carrot cells, cultured in the presence of the synthetic auxin 2,4-D, proliferate in an undifferentiated state, whereas removal of 2,4-D induces somatic embryogenesis. In this system, IAA is synthesized at all times; however, two different biosynthetic pathways are utilized in a developmentally or 2,4-D-regulated manner. Cells proliferating in the presence of 2,4-D synthesized IAA from Trp. Once 2,4-D was removed and the cells were undergoing embryogenesis, Trp was no longer utilized as a precursor and instead IAA was produced via a Trp-independent pathway (Michalczuk et al., 1992). This is the first clear demonstration of multiple IAA biosynthetic pathways in a single plant type, and it indicates a greater degree of regulatory complexity than has been previously presumed.

There is evidence for multiple pools of Trp throughout the cell and IAA biosynthesis has been shown to occur in chloroplasts as well as in the cytoplasm (reviewed by Nonhebel et al., 1993). For this reason it has been argued that lack of incorporation of label from Trp into IAA could be due to the inability of the amino acid to localize to the site of IAA biosynthesis. The experiments carried out in the carrot system demonstrated that deuterated Trp labels plastids and cytoplasm with similar efficiency (Michalczuk et al., 1992), an important point for interpreting in vivo labeling studies.

Although recent work has provided an exciting impetus to the field of IAA biosynthesis, we are still left with the same question: how do plants make IAA? Both the maize endosperm and carrot cell culture systems will be useful in the characterization of the novel Trp-independent pathways. Recently, Bandurski and co-workers demonstrated the suitability of immature maize endosperm as a model system to study IAA biosynthesis. In this in vitro system radioactive indole was converted into IAA, indicating that all of the enzymes required for IAA biosynthesis must be present (Rekoslavskaya and Bandurski, 1994). Furthermore, Trp does not appear to be the precursor to IAA, since the yield of radioactive IAA from labeled indole was not reduced by the addition of unlabeled Trp (Jensen and Bandurski, 1994). These results are consistent with the in vivo data from the *orange pericarp* mutant.

Those systems in which Trp is confirmed to be the precursor to IAA can now be examined much more quantitatively with isotope dilution analysis to determine which of the previously postulated pathways from Trp are utilized. Below is a summary of those pathways (see Fig. 1).

The Indole-3-Pyruvate Pathway

Nonhebel and Cooney used isotope dilution analysis to establish that indole-3-pyruvate is present as a natural component of tomato (Cooney and Nonhebel, 1991; Nonhebel et al., 1993). In deuterium-labeling studies with seedlings, indole-3-pyruvate incorporated more deuterium than IAA, which would be expected of a precursor to IAA. Because incorporation of deuterium into Trp was lower than into indole-3-pyruvate, Nonhebel and Cooney invoke Trp compartmentation and selective utilization of Trp pools to explain the observed labeling patterns. In light of recent results in other systems, however, their data are not inconsistent with a Trp-independent IAA biosynthetic pathway. Although enzyme activities for each step in the indole pyruvate pathway have been identified in plants, and the genes encoding indole-pyruvate decarboxylase have been isolated from *Enterobacter cloacae* (Koga et al., 1991) and *Azospirillum brasilense* (Costacurta et al., 1994), none of the plant genes encoding these enzymes has been cloned.

The IAN Pathway

IAN and other components of the IAN pathway have been found primarily in the Brassicacae; therefore, this pathway has not been considered to be of general importance. Trp is converted to indole-3-acetaldoxime by an enzyme activity that has been detected in several plant species (Ludwig-Müller and Hilgenberg, 1988). Indole-3-acetaldoxime conversion to indole-3-acetonitrile has been demonstrated in plasma membranes of Chinese cabbage (Ludwig-Müller and Hilgenberg, 1990). The nitrilase that converts indole-3-acetonitrile to IAA has been cloned in *Arabidopsis* (Bartel and Fink, 1994; Bartling et al., 1994), where there are four genes that show differential expression (Bartel and Fink, 1994). Because *Arabidopsis* is particularly amenable to molecular genetic analysis, this pathway has received renewed interest. Although earlier studies suggest that IAN is derived from Trp, IAN accumulates in the *Arabidopsis trp2* mutant, indicating that it could be derived independently of Trp (Normanly et al., 1993).

The majority of IAA in plants is conjugated to sugars, peptides, amino acids, or *myo*-inositol via ester or amide linkages. The identities of IAA conjugates are at present unknown in most plant species, so in order to quantify ester-linked and amide-linked IAA, extracts are treated with base to cleave the conjugates and yield free IAA, which is then fractionated on HPLC. The Brassicacae produce a large variety and quantity of indole compounds, IAN and indoleglucosinolates among them. These compounds present a challenge in the quantitation of IAA, since they can be potentially converted nonenzymatically to IAA in alkaline conditions. Therefore, when measuring conjugated IAA in *Arabidopsis* or other Brassicacae, it is necessary to account for the "background" levels of IAA that are derived from nonenzymatic conversion of IAN. This entails adding ^{13}C-labeled IAN as an internal standard to determine the amount of IAN in the sample under conditions of neutral pH, then subtracting the ^{13}C-labeled IAA and corresponding amounts of unlabeled IAA that result from breakdown of IAN (Normanly et al., 1993). Selective hydrolysis applied to members of the Brassicacae without correcting for IAN conversion is subject to significant error. Under the standard conditions used for extraction of conjugated IAA, indolemethylglucosinolate does not convert to IAA. Knowing the identity of IAA conjugates in *Arabidopsis* would simplify quantitation enormously, since they could then be analyzed directly.

The Tryptamine Pathway

The first step in a third pathway postulated for the conversion of Trp to IAA involves the decarboxylation of Trp to tryptamine by way of TDC. The gene encoding this enzyme has been isolated from *Catharanthus roseus* (De Luca et al., 1989) and tryptamine has been identified as a native compound in tomato by GC-MS (Cooney and Nonhebel, 1991). Transgenic tobacco expressing the *C. roseus* TDC gene under control of the cauliflower mosaic virus 35S promoter accumulated tryptamine but not IAA (Songstad et al., 1990). This appears to negate a role for tryptamine in IAA biosynthesis, although it could be argued that the 35S promoter did not direct expression of TDC in a manner that was temporally and spatially compatible with the other enzymes in this pathway. Deuterium labeling ruled out tryptamine as an intermediate in tomato (Cooney and Nonhebel, 1991), and tryptamine is not universally present in plants; thus, a tryptamine pathway may not be widespread.

D-Trp versus L-Trp

Radiolabeling studies, together with the observation that D-Trp stimulated seedling growth more effectively than L-Trp, led several laboratories to the hypothesis that the D- rather than the L-isomer of Trp is used as the IAA precursor (see Baldi et al., 1991). This theory was supported by the report that 4-Cl-Trp, the expected precursor to 4-Cl-IAA found in pea, also occurred in the D-form (see Sakagami et al., 1993). Baldi et al. (1991) carried out a careful labeling study using *Lemna* as a model system to test this premise and found no evidence for such a pathway. The *Lemna* experiments were performed under aseptic conditions, and uptake of both D- and L-forms of Trp from the medium occurred rapidly. Even after several days, the D-[^{15}N]Trp taken up from the medium was not converted into [^{15}N]IAA, although there was a several hundred-fold enrichment of the D-Trp pool. In addition, only low levels of L-Trp conversion were observed, and this L-[^{15}N]Trp to [^{15}N]IAA labeling occurred without detectable labeling of the D-Trp pool (Baldi et al., 1991).

Conversion of *N*-malonyltryptophan (found in vivo, in both the L- and D-Trp forms) to indole-3-acetaldoxime and then to IAA has been proposed as another route to IAA. However, Ludwig-Müller and Hilgenberg (1989) showed that whereas *N*-malonyltryptophan was converted, it was *N*-malonyl-L-tryptophan that was the substrate for this reaction. An additional set of data that is not widely known but that also sheds light on this area shows that 4-Cl-Trp, the expected precursor to 4-Cl-IAA in pea seeds, occurs primarily in the L-form. Contrary to previous reports, only about 2% of 4-Cl-Trp is in the D-form and the bulk of 4-Cl-Trp is in the L-isomer (Sakagami et al., 1993). These results suggest that only L-Trp can be converted into IAA.

OTHER AUXINS

Although IAA was the first auxin isolated and is the major auxin, other compounds with auxin activity occur in plants as well. Most of these compounds are active only at higher concentrations than IAA and their role in growth remains largely unknown. IBA and 4-Cl-IAA are two indolic auxins other than IAA with significant biological activity. IBA has recently been positively identified in plants by GC-MS (Epstein and Ludwig-Müller, 1993). The role of IBA in plant growth regulation is unknown, although it is implicated in root formation and widely used commercially for induction of adventitious rooting. The interconversion of IBA and IAA occurs in plants invoking a mechanism of chain lengthening and β-oxidation, analo-

gous to that occurring in fatty acid biosynthesis. Nothing is known about the regulation of such reactions.

A highly active halogenated indole auxin, 4-Cl-IAA, has been identified in a number of plants, mainly members of the Fabaceae, but also in pine seeds (Ernstsen and Sandberg, 1986, and refs. therein). In bioassays, 4-Cl-IAA has been shown to have up to 10 times the biological activity of IAA. 4-Cl-IAA occurs as the methyl ester in many of the plants examined, although 4-Cl-IAA aspartate and its monomethyl ester have also been described. As with IBA, a clear physiological role for 4-Cl-IAA has not been established, although the recent report of its activity in the stimulation of pod growth in deseeded pea, where other auxins are weak or inactive, and its presence in seeds and pod tissue suggest a function in pod development (Ozga et al., 1993).

WHY NO AUXIN AUXOTROPHS?

Mutants defective in biosynthesis of such a major hormone might very well be inviable. The discovery of multiple IAA biosynthetic pathways provides an additional explanation for the lack of auxin auxotrophs. The interdependence of these pathways may be the real reason for the lack of auxin biosynthetic mutants. Are these pathways separable in space and time? Would a block in one pathway result in compensation by another pathway? Data regarding these issues are accumulating as a consequence of the newly available genetic and quantitative tools, but explanations are still only working hypotheses.

IS IAA CONJUGATION A KEY POINT OF REGULATION?

In mutants or transgenic plants that accumulate IAA, the levels of free IAA generally remain normal while conjugated IAA accumulates. This leads to the speculation that free IAA is the biologically active form and that conjugation of IAA is a control mechanism for hormone levels. The IAA-conjugating and -deconjugating enzymes are therefore of great interest from a regulatory standpoint. Hangarter and Good (1981) showed that auxin conjugates could be used as "slow-release" forms of IAA in plant tissue cultures. They attributed differences in physiological activity and persistence to the slow hydrolysis of IAA amino acid conjugates, although hydrolysis rates were not measured. The in vivo hydrolysis of IAA amino acid conjugates was studied by applying radioactive conjugates to bean stems and measuring the release of free IAA by reverse isotope dilution analysis (Cohen et al., 1988, and refs. therein). The rate of hydrolysis was found to correlate positively with stem bending. Conjugate hydrolysis has been extremely difficult to reproduce in vitro, thereby hindering the isolation of these enzymes. An extract capable of hydrolyzing IAA-amino acid conjugates was prepared from bean tissue (Cohen et al., 1988), but the activity was too labile for purification. Kuleck and Cohen (1992) reported the isolation of a similar, also labile, enzymatic activity from carrot cell cultures. This enzyme showed specificity for IAA-Ala, IAA-Phe, and related amino acid conjugates. A report that the protein encoded by the *Agrobacterium rhizogenes rolB* gene might catalyze the hydrolysis of auxin conjugates has now been shown to be incorrect (Nilsson et al., 1993).

The pathways for auxin conjugation have been studied most extensively in the endosperm of maize kernels (for review, see Bandurski et al., 1994), where esters among IAA and inositol, Glc, inositol glycosides, and glucans account for essentially all of the IAA present. Enzymes that catalyze the following reactions have been described, and in some cases at least partially purified:

$$\text{IAA} + \text{UDP-Glc} \leftrightarrow \text{1-}O\text{-IAA-Glc} + \text{UDP}$$

$$\text{1-}O\text{-IAA-Glc} + \textit{myo}\text{-inositol} \rightarrow \text{IAA-}\textit{myo}\text{-inositol} + \text{Glc}$$

$$\text{IAA-}\textit{myo}\text{-inositol} + \text{UDP-Gal} \rightarrow \text{IAA-}\textit{myo}\text{-inositol-Gal} + \text{UDP}$$

$$\text{IAA-}\textit{myo}\text{-inositol} + \text{UDP-Ara} \rightarrow \text{IAA-}\textit{myo}\text{-inositol-Ara} + \text{UDP}$$

$$\text{1-}O\text{-IAA-Glc} \rightarrow \text{IAA} + \text{Glc}$$

$$\text{IAA-}\textit{myo}\text{-inositol} + \text{Glc} \rightarrow \text{6-}O\text{-IAA-Glc}$$

$$\text{6-}O\text{-IAA-Glc} \rightarrow \text{IAA} + \text{Glc}$$

In addition, in vitro evidence exists for the conversion of IAA-*myo*-inositol-Ara and IAA-*myo*-inositol-Gal back to IAA-*myo*-inositol. The first gene for a plant enzyme involved in IAA metabolism to be cloned is the maize gene for the IAA-Glc synthase (Szerszen et al., 1994). Tobacco plants overexpressing this gene showed reduced apical dominance and weak geotropism but normal flowering.

A novel peptide conjugate has been identified in bean seedlings (Bialek and Cohen, 1986). Antibodies raised against the 18-amino acid peptide can be used to determine the prevalence of this conjugate in other plant species. Cloning the gene encoding this peptide will be useful for investigating the role of protein-IAA conjugates in plants and the regulation of peptide conjugation.

A number of IAA-induced genes show significant homology to GST. A 25-kD polypeptide with significant homology to tobacco and maize GST was isolated from *Hyoscyamus muticus* based on its binding to azido-IAA (Bilang et al., 1993). This protein had GST activity and in fact could be purified based on its affinity for glutathione. In competition assays active auxins were able to inhibit labeling of this GST by azido-IAA, giving rise to speculation that GST may play a role in IAA metabolism. It has been speculated that GST facilitates the formation of the conjugate IAA-Glc via an IAA-CoA intermediate. The recent isolation and cloning of the IAA-Glc synthase precludes this possibility, however, since this enzyme does not utilize IAA-CoA as an intermediate and the gene has homology to a Glc transferase (Szerszen et al., 1994).

IAA TURNOVER

The level of IAA available to mediate a biological response in a cell at any given time is regulated by a number

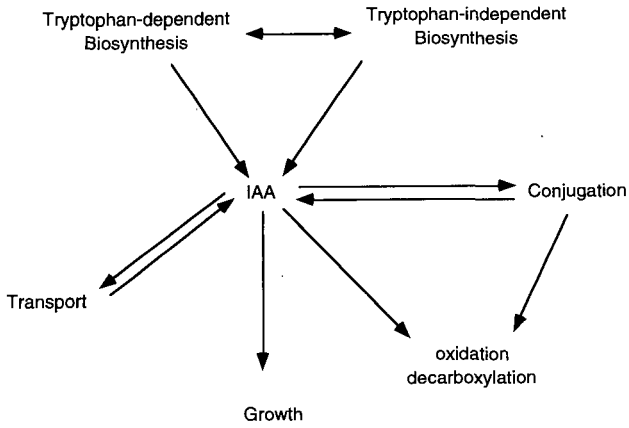

Figure 3. Factors affecting IAA levels in plant cells.

of factors: biosynthesis, conjugation, transport, and degradation (Fig. 3). While biosynthesis and conjugation have been the focus of intense study with recent breakthroughs, degradation or "turnover" has been largely ignored until recently. The isolation of α-methyl-Trp-resistant lines of *Lemna* is providing new insight into the importance and dynamic nature of IAA turnover (Tam et al., 1995). In these lines, anthranilate synthase is resistant to feedback inhibition by Trp, which results in Trp accumulation. Indole and indole-3-glycerol phosphate might also be expected to accumulate and lead to increased IAA levels, yet GC-MS analysis showed only a slight increase in free IAA and no change in conjugated IAA levels. Most interestingly, the half-life of IAA was 1 h, 10 times faster than that of wild type. Somehow, degradation of IAA has increased in response to the greater flux of metabolites through the Trp biosynthetic pathway. With the sensitivity afforded to IAA analysis by GC-MS, it should now be possible to make a thorough examination of IAA turnover as a function of developmental and environmental state.

Whatever Happened to IAA Oxidase?

It is also important to note that our ideas concerning IAA catabolism have recently undergone substantial revision. IAA catabolism was thought to occur primarily through the action of IAA oxidase, a companion activity to most peroxidases of plant origin. This concept has now received serious challenge in that (a) the products of "IAA oxidase" (noted by the loss of the carboxyl carbon) do not appear to be present in plants in significant amounts (Ernstsen et al., 1987), and (b) experiments with transgenic plants show no change in IAA levels even with a 10-fold increase in peroxidase expression or a 90% decrease in peroxidase levels (Lagrimini, 1991).

Recent data provide evidence for two nondecarboxylative oxidation routes. In the first, IAA is oxidized to oxindole-3-acetic acid and subsequently glycosylated through an added 7-OH. An enzyme from maize has been isolated that oxidizes IAA at the 2 position in a nondecarboxylating manner, and this product, oxindole-3-acetic acid, is found in plants in quantities similar to that of IAA (Reinecke, 1990). In a second pathway, IAA is conjugated to aspartate and subsequently oxidized (Tsurumi and Wada, 1990). The resultant oxindole-3-acetylaspartate can either be further oxidized to the 3-hydroxy derivative or hydrolyzed back to oxindole-3-acetic acid (Tuominen et al., 1994). Recent progress in this area highlights the need to study turnover as a component of IAA regulation.

CONCLUSIONS

Within the last 10 years the basic assumptions about how indolic auxin compounds are made and degraded in plants have changed. These changes have created new challenges to our understanding of how auxins regulate development in plants and how, in turn, development regulates the rates of production, rates of degradation, type of indolic auxin, and even the pathways that will be used to produce these compounds.

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Investigating Auxin Metabolism in Arabidopsis thaliana Mutants With Altered Adventitious Rooting via High Throughput Indolealkanoic Acid Quantification

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INVESTIGATING AUXIN METABOLISM IN *ARABIDOPSIS THALIANA* MUTANTS WITH ALTERED ADVENTITIOUS ROOTING VIA HIGH THROUGHPUT INDOLEALKANOIC ACID QUANTIFICATION

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ABSTRACT

Auxins are a class of plant hormones, or phytohormones, that mediate the coordination of a number of important growth and behavioral processes in plants. The two widely distributed naturally occurring auxins are indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA). Interestingly, IBA can be converted to IAA and IAA can serve as a precursor to IBA. These two events happen through two different pathways. IAA is converted into IBA in a two-step process that is only partially understood. This work aims to identify by genetic analysis the unknown enzyme involved in catalyzing the initial step in this biosynthetic conversion of IAA to IBA through the quantification of these auxins within lines of *Arabidopsis thaliana* mutated in genes that have been positively or negatively correlated with endogenous auxin levels and/or developmental events mediated by these hormones, such as adventitious root formation. Endogenous IAA and IBA were extracted via an automated high-throughput solid-phase extraction method and a modified post-extraction clean-up specifically designed to purify both IAA and IBA within the same sample. GC-SIM-MS analysis of samples utilized a novel IBA internal standard, [$^{13}C_8{}^{15}N$]-indole-3-butyric acid. This modified high-throughput method of multiple indolealkanoic acid quantification was applied to determine IAA and IBA concentrations in different ecotypes of *Arabidopsis thaliana* seedlings under different experimental growth conditions. Once optimal growth conditions for IBA production were identified, IBA and IAA levels in a series of insertional mutants revealed two *gh3-6* mutants with an increased ratio of IAA to IBA concentrations suggesting that the GH3-6 protein is involved in maintaining endogenous levels of IAA and IBA.

INTRODUCTION

Introduction to the Phytohormone Class of Auxins

Auxins are a class of plant hormones, or phytohormones, that are involved in nearly every developmental event within plants, from embryogenesis to senescence (Slovin et al., 1999). Auxin compounds are classified by their demonstrated ability to induce particular developmental events such as increasing the rate of cell elongation, increasing lateral root production, inhibiting root elongation (Zolman et al., 2000), inducing adventitious root formation (Zimmerman and Hitchcock, 1942), and the bending of pea explants (Fawcett et al., 1960) when applied to plants. Early bioassays revealed four major compounds that exert auxin-like effects: indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D), and naphthalene acetic acid (NAA) (Woodward and Bartel, 2005). Where IAA and IBA are primary auxins produced in plants, NAA and 2,4-D are synthetic auxins widely used as plant growth regulators or as auxinic herbicides. Both IAA and IBA are defined as indolealkanoic auxins because of their shared indole group and carboxylic acid chain extending from the C3 in the indole ring. Other auxins that have been found in plants include a chlorinated form of IAA, 4-Cl-IAA (Slovin et al., 1999), phenylacetic acid (PAA) (Ludwig-Müller and Cohen, 2002) as well as two IAA precursors indole-3-pyruvic acid (IPA) and indole-3-acetonitrile (Thinmann, 1977). Figure 1 shows the chemical structure of these common auxins.

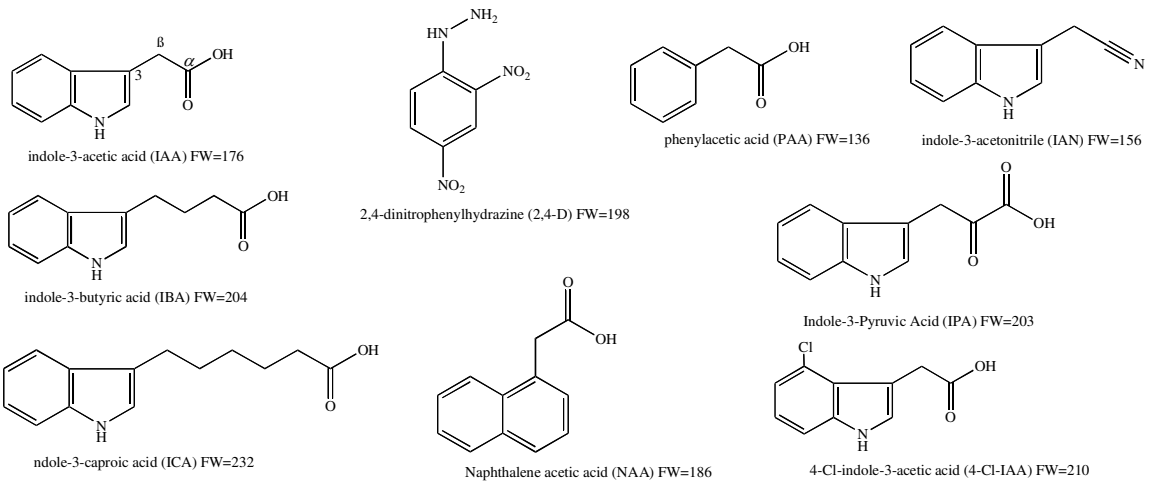


Figure 1 *Chemical structures of compounds that have auxin activity.* All of the compounds listed above, with the exception of ICA, have been identified within *Arabidopsis*.

Much attention in phytohormone research has been given to IAA as it exerts direct control over hormone response genes (Reed, 2001), is indicated in a myriad of developmental processes (Davies, 1995), and exists in quantifiable amounts within *Arabidopsis thaliana* (Ilic et al., 1996), pea (Schaerer and Pilet, 1993), bean plants (Bialek and Cohen, 1989), *Oryza sativa* (Kobayashi et al., 1989), *Zea Mays* (Pilet and Saugy, 1987) and Douglas firs (Crozier et al., 1980), among others. Combined gas chromatography-mass spectrometry (GC-MS) analysis has demonstrated the presence of IAA in numerous plant tissues at concentrations ranging from 1-1000 ng/g fresh weight (Sandberg et al., 1987). While the physiological responses to auxins are well characterized, our understanding of the exact genetic and molecular mechanism of auxin metabolism, regulation, and signaling remains incomplete.

In general, hormonal control within plants is neither hierarchical nor mutually exclusive to specific phytohormones. Rather, discrete physiological activities mediated by hormones require multiple hormonal inputs to ensure appropriate responses. However, certain classes of phytohormones exert greater control over particular

development events. Auxins exert control over developmental and behavioral responses within plants through complex interactions between numerous auxin compounds (Zolman et al., 2000) and with other phytohormones such as gibberellins (Fu and Harberd, 2003; Olszewski et al., 2002), abscisic acid, cytokinin, ethylene and jasmonate (Nemhauser et al., 2006). One important phytohomone relationship within plants is between the two primary auxins IAA and IBA.

Auxins mediate dynamic adaptive response to changes within a plants environment such as drastic temperature changes, light and dark cycles, and exposure to pathogens and other biotic and abiotic stresses. Growth retardation and reduced metabolic rates are symptomatic of infected or otherwise stressed plants. These responses are mediated by the reallocation of metabolic resources between different pathways that maximize plant survival under stress conditions. One of the primary ways in which plants coordinate auxin-mediated processes that guard against stresses is to maintain the endogenous pool of the primary auxin, IAA, at an appropriate level by regulating auxin biosynthesis and distribution among different organs and by conjugate formation with sugars, peptides, and amino acids (Park et al., 2007).

Higher plants have devised at least two ways of controlling the endogenous pool of IAA that does not fully catabolize IAA in order to store IAA for later use; the formation of IAA-amino acid conjugates and through the production of IBA. Amino acid conjugates (such as IAA-Asp, IAA-Glu, and IAA-Ala) provide free IAA upon hydrolysis. Alternatively, IBA can be converted to IAA in peroxisomes in a process similar to fatty acid ß-oxidation (Zolman et. al., 2000). IAA conjugates and IBA are involved in IAA storage, transport, compartmentalization and provide a mechanism to sequester excess

IAA to safeguard against toxic IAA levels and further protects IAA against peroxidative degradation (Cohen and Bandurski, 1982).

Two important studies published within the past year that highlight these distinct mechanisms involved in auxin homeostasis that are essential elements regulating stress adaptation responses. Park et al. (2007) showed that an auxin-responsive *GH3* gene encoding a IAA-amino acid conjugation enzyme is induced by various biotic and abiotic stresses, suggesting that GH3-mediated growth suppression directs reallocation of metabolic resources to establish stress resistance. Ludwig-Müller (2007) demonstrated that IBA synthetase activity increased dramatically in maize seedlings when subjected to drought conditions. Together, these observations present two potential pathways of IAA homeostasis that achieve a similar result: the reduction of endogenous IAA. In one instance through the reduction of amino acid conjugation, in the other, IAA is reduced as it is converted to IBA. These works provide the necessary background in elucidating the biosynthesis of IBA from IAA as they propose that beyond the developmental importance of IBA within normal growth processes, IBA is produced in response to stresses in order to maintain IAA homeostasis. Furthermore, these observations suggest that beyond maintaining homeostasis changes with IBA and IAA concentrations within plants generate specific stress responses.

Indole-3-Butyric Acid (IBA): History and Function

IBA was first identified as a "synthetic hetero-auxin" that induced increased rooting in lemon and Chrysanthemum cuttings (Cooper, 1935). Thus the initial classification of IBA was a synthetic auxin able to induce the heartiness of root cut

species far better than naturally occurring auxins through its ability to initiate rooting (Zimmer and Wilcoxon, 1935). It wasn't until 1954 that IBA was tentatively reported as a natural product through the analysis of acids extracted from potato tuber peels and exposing explants to these extracts and observing root formation (Bommaert, 1954). This research was an early demonstration of the importance of applying sensitive chemical assays to purify and identify auxins.

Within the past fifteen years advances in molecular genetic and chemical techniques, such as combined gas chromatography-mass spectrometry (GC-MS) established that IBA is an endogenously produced compounds in a variety of species that plays an indispensable role within plant physiology (Ludwig-Muller, 2000; Woodward and Bartel, 2005). Table 1 lists the species IBA has been detected via gas chromatography-mass spectrometry in chronological order of their discovery.

Plant Species	**Organ**	**Reference**
Pisum Sativum	Root nodules	Badenoch-Jones et al. 1984
Pisum Sativum	Roots, epicotyls, cotyledons	Schneider et al. 1985
Cupressus sp.	Leaves	Epstein et al. 1989
Zea Mays	Leaves, kernals	Epstein et al. 1989
Zea Mays	Root inoculated with *Azospriillum*	Fallik et al. 1989
Zea Mays	Roots, leaves, coleoptiles	Ludwig-M□ller and Epstein 1991
Daucus carotia	Hairy root	Epstein et al. 1991
Nicotiana tobacum	Leaves	Sutter and Cohen 1992
Arabidopsis thaliana	Seedlings	Ludwig-Muller et al. 1993
Zea Mays	Leaves, roots, AM-inoculated roots	Ludwig-Muller et al. 1997
Tropaeolum majus	Roots, shoots leaves, flowers	Ludwig-Muller and Cohen, 2002

Table 1 *Identification of indole-3-butyric acid as an endogenous compound in different plant species by gas chromatography-mass spectrometry (GC-MS).* Quantification of IBA within these studies relied on either [^{3}H]-IBA, [$^{13}C_1$]-IBA internal standard.

In general, IBA is present in equal or lower concentrations than IAA and represents, when present, between 25% and 30% of the total free auxin pool in *Arabidopsis* (Ludwig-Müller et al., 1993). The occurrence of IBA varies between species (Ludwig-Müller, 2000) and among different ecotypes and cultivars (Ludwig-Müller 2007; Dunberg et al. 1981; this research). It has been demonstrated that IBA is present at

different concentrations within different parts of the plant (Epstein et al., 1989) and its levels apparently fluctuates throughout development (Ludwig-Müller, 2007). IBA levels are highly dependent upon growth conditions such as pH, volume of culture flask (which effects gaseous ethylene levels), and light intensity (Ludwig-Müller, 2007). Furthermore, it has been postulated that IBA does not occur in some cultivars of the same plant species (Epstein et al., 1989). However, this lack of detection does not rule out the presence and activity of IBA because the techniques employed at quantifying IBA were not sensitive enough to detect the low concentrations of IBA with confidence.

Indeed, more sensitive methods of quantifying IBA are needed to gain a more complete understanding of the function and regulation of IBA within plants. It is no surprise that IBA evaded researchers for as many years as it has. Not only can it naturally exist at extremely low concentrations, it has proven difficult to design experimental growth conditions to maximize its concentrations. To date, as reviewed by Ludwig-Müller (2000), there has been only limited research aimed at establishing the impact of IBA in plants through the development of quantification methods.

Indole-3-butyric Acid Transport

Important in understanding the diversity of the biological activity of IBA is determining how plants transport this compound. Like IAA, IBA is thought to be produced in specific regions of plants, such as the apical region of leaves, and subsequently transported throughout the plant using a series of transport proteins.

Investigations into polar auxin transport have led to the identification of a series of efflux carrier membrane proteins involved in polar IAA transport know as PIN

proteins (Galwëiler et al., 1998; reviewed by Friml and Palme, 2002). This work led to the interesting work surrounding the hypothesis that the polar transport of IBA follows a different pathway than IAA. IBA transport is not sensitive to IAA efflux inhibitors and IAA, not IBA, transport is disrupted in plants that have mutations in genes responsible for these PIN IAA efflux carriers (Rashotte et al., 2003). Rashotte et al. (2003) further showed that both IAA and IBA transport approach a saturation point as an increasing amount of synthetic hormone were applied suggesting distinct mechanisms of protein-mediated polar transport for both IBA and IAA. These intriguing results suggest that not only does the transport of IBA involved specific carriers, but it is also the strongest evidence to support the hypothesis that IBA has activities beyond being a so-called "slow-release" form of IAA. That is, if IBA transport to specific tissues doesn't result in the production of IAA it would appear that IBA is having some physiological effect on its own. However, it is important to note that the tissues examined for polar auxin transport by this group displayed similar transport kinetics as both IBA and IAA under certain light/dark growth conditions implying that there could be overlap in their transport systems under specific conditions.

Important work has yet to be done to effectively assess the differential transport of IAA and IBA within tissues known to be more dependent upon polar IAA transport. The hypothesis that IBA acts as an independent auxin as suggested by Rashotte et al. (2003) is supported by these observations that assess IBA transport in the IBA-unresponsive *resistant to IBA* (*rib1*) mutant in Arabidopsis (Poupart et al. 2005). They observed that IBA, not IAA, transport was decreased dramatically in the *rib1* mutant, suggesting a possible family of proteins involved in IBA polar transport. That is, the

authors hypothesize that the primary reason why *rib1* does not respond to IBA is because they lack elements of the polar transport system that deliver IBA to tissue where it is most active.

Indole-3-Butyric Acid Metabolism

Exactly how IBA acts at the molecular level is unknown. An important route that IBA exerts its auxin effects through its conversion to IAA. Genetic and biochemical evidence has revealed that the conversion of IBA to IAA is similar to peroxisomal fatty acid synthesis. Recently, Zolman et al. (2007) identified an IBA-responsive mutant *ibr3* with decreased responses to the inhibitory effects of IBA on root elongation or the stimulatory effect on lateral root formation while responding normally to IAA application suggesting that IBR3, an acyl-CoA dehydrogenase with a perioxsomal targeting sequence, is essential in the conversion of IBA to IAA. IBA has been thought to function as a so-called "slow release" form of IAA that behaves similarly to IAA conjugates (van der Krieken et al., 1997). Because IAA and IBA share a common indole group it has been possible to radiolabel carbons in this ring within IBA, 'feed' it to plants, and observe the generation of radiolabeled IAA, confirming that IBA was converted to IAA (van der Krieken et al., 1992). Alternatively, it has been shown that IAA acts as a precursor to IBA through a series of chain elongation reactions similar to that of fatty acid synthesis (Ludwig-Müller, 2000).

It has been rationalized that the reason synthetically produced IBA is more effective at inducing auxin effects is that IBA is a more stable compound, having a half-life over four times as long as IAA in solution and can therefore enter into tissues more

effectively than IAA to then be converted to IAA *in planta* inducing root formation (Nordström et al., 1991). If this hypothesis were correct, endogenous levels of IAA would increase proportionately to the application of synthetically produced IBA. This, however, has been found not to be the case: high levels of applied IBA do not lead to a considerable increase in endogenous IAA concentrations (Ludwig-Müller, 2000). Thus, it appears that IBA alone has some direct responsibility for root growth induction.

Like IAA, plants have devised ways to metabolize IBA by forming conjugates, increasing their stability for storage. In *Arabidopsis* where IAA is found primarily as amide conjugates to amino acids, ester conjugates of IBA to glucose dominates in this plant (Woodward and Bartel, 2005). However, some experimental evidence has shown that IBA can be effectively metabolized to both ester and amide conjugates, primarily in the form of IBA-Asp (Baraldi et al., 1993).

Biosynthesis of Indole-3-Butyric Acid

The biosynthesis of IBA could follow at least three pathways: (1) via a tryptophan-dependent pathway, (2) tryptophan-independent pathway similar to the biosynthetic pathways responsible for IAA generation (see Wright et al. 2002; Cohen et al., 2003), or (3) via a chain elongation reaction analogous to fatty acid synthesis using IAA as its substrate (Ludwig-Müller, 2000). However, the latter is the only pathway strongly supported by experimental evidence.

Important work has been done in mapping the IBA biosynthetic pathway and identifying enzymes that catalyze these reactions. However, as will be shown, elements that converts IAA to IBA, two compounds that share a high degree of structural similarity

differing by only two carbons extending from an indole ring, remain to be determined. Figure 2 outlines the biosynthetic relationship between IAA and IBA.

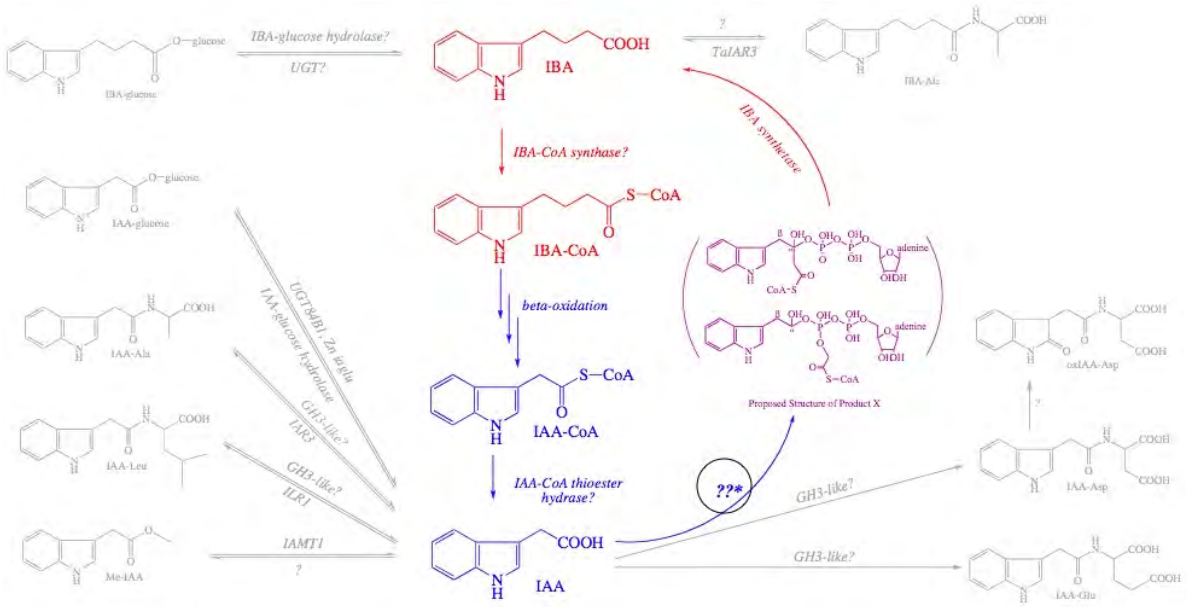


Figure 2 *Potential pathways of IAA and IBA metabolism and biosynthesis.* IAA and IBA can be interconverted through independent mechanism. Red, blue, and purple structures are IAA, IBA, and intermediate structures. The exact structure of 'product X' is unknown. The circled *??** denotes the step within the conversion of IAA to IBA that is the focus of this research. Suggested conversion for which plant genes are not identified are indicated with question marks. Structures of product X are adapted from Ludwig-Müller et al. (1995b) and over all metabolic relationships are adapted from Woodward and Bartel (2005).

Investigations into the *in vitro* conversion of IAA to IBA in *Zea mays* revealed that IBA formation is dependent on four components, a microsomal membrane preparation, IAA, ATP, and acetyl CoA (Ludwig-Müller et al.1995b). The reaction is described as the acetylation of the IAA carboxylic group with acetyl-CoA that results in the addition of two more carbons on the carboxylic acid chain extending from the indole ring that differentiate IBA from IAA. This work in addition to a follow up study (Ludwig-Müller and Hilgenberg, 1995a) revealed that this conversion reaction is a two-step process catalyzed by two different enzymes. Incubation of microsomal membranes

from the roots and hypocotyls of dark-grown maize with IAA, acetyl CoA and ATP resulted in the formation of an as yet to be identified reaction intermediate known as 'product X' that proceeds to form IBA in the presence of the cofactor NADPH (Ludwig-Müller et al. 1995b). The second step in this reaction, the formation of IBA from the reaction intermediate, has been shown to be catalyzed by a 31 kDa enzyme known as IBA synthetase. The formation of the reaction intermediate is reportedly catalyzed by a 20 kDa yet to be purified and identified enzyme that is associated with different membrane fractions than IBA synthetase (Ludwig-Müller 1995a). This work conducted more than 10 years ago has led to some important insight in IBA activity. However, it also represents a loose end in IBA biochemistry in that it only partially describes the biosynthesis of IBA.

The endogenous concentration IBA correlates with IBA synthetase activity (Ludwig-Müller, 2007). This suggests that although there may be other possible routes plants used to synthesize IBA, utilizing IAA as a precursor is perhaps the dominant pathway. However, this does not rule out the possibility that plants may employ other biosynthetic pathways to IBA in different tissues or at specific developmental stages. Given that IBA is widely considered a storage derivative of IAA, its regulation is likely dependent upon external conditions that trigger the plant to sequester IAA for later use such as the conjugation of IAA to amino acids via GH3 proteins in response to stresses. However, it appears that the conversion of IBA from IAA is more than a way in which a plant can maintain appropriate IAA levels for later use. Ludwig-Müller (2007) showed that IBA synthetase activity sharply increased in maize seedlings when subjected to drought stress conditions. As noted by these authors, changes in IBA synthetase activity

were accompanied by changes in root morphology typical to the maize drought response including the thickening of the elongation zone, an increase in the number of root hairs, and the curling of the roots. Based on these observations, they suggest that one natural role of IBA might be the induction of changes in root morphology and the number of roots during stress (Ludwig-Müller, 2000). Thus, it appears that the conversion of IBA from IAA plays a distinctive role in stress response from that of GH3-mediated events that lead to the production of stable storage conjugates.

Formation of product X

The exact structure of the reactive intermediate, product X, and the enzyme that catalyzes the addition of the acetyl CoA and ADP (via ATP) have yet to be determined. Preliminary chemical analysis of product X has revealed two possible structures; see Figure 3.

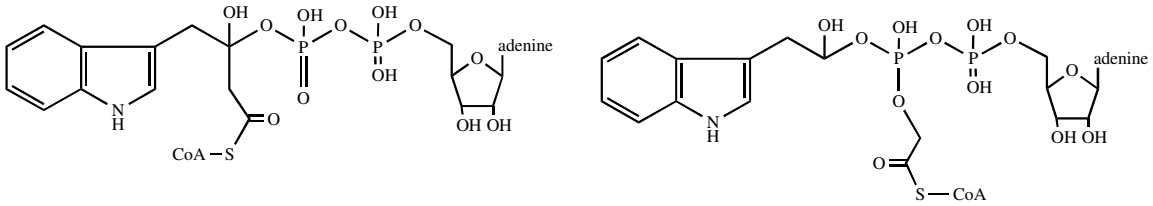


Figure 3 *Proposed chemical structures of the intermediate structure of the biosynthesis of IBA from IAA, product X.* Adapted from Ludwig-Müller (1995a).

This intermediate is characterized by the conjugation of ADP and acetyl-CoA with IAA. Understanding the structure of this intermediate is key in the search for the enzyme that catalyzes this reaction. These provocative findings suggest that this first step within the biosynthesis of IBA proceeds via an ATP catalyzed ADP-IAA intermediate in which the α carbon of IAA is adenylated.

These observations provide a starting point in the search for enzymes that could potentially be responsible for this reaction. Broadly, such an enzyme would be an ATP-dependent acetyl-CoA transferase. The mechanism proposed by Ludwig-Müller (1995a) is strikingly similar to the mechanism of IAA amino acid conjugation that is characterized by the formation of an AMP-IAA intermediate, a reaction catalyzed by GH3 proteins (Staswick et al., 2005) differing by the addition of AMP rather than ADP.

Studies of mutants responsive to jasmonic acid, a phytohormone structurally similar to indolealkanoic acids, demonstrated that a family of enzymes known collectively as *GH3* genes (Gretchen Hagen, named after the researcher who first characterized this family), originally identified as an auxin-response family of genes (Hagen and Guilfoyle, 1985), conjugates amino acids to both jasmonic acid and IAA (Staswick et al., 2002; Staswick et al., 2005). Phylogenic analyses of this family of 20 genes reveal three distinct groups that appear to have divergent functions (Staswick et al., 2005 and this work; see Figure 4).

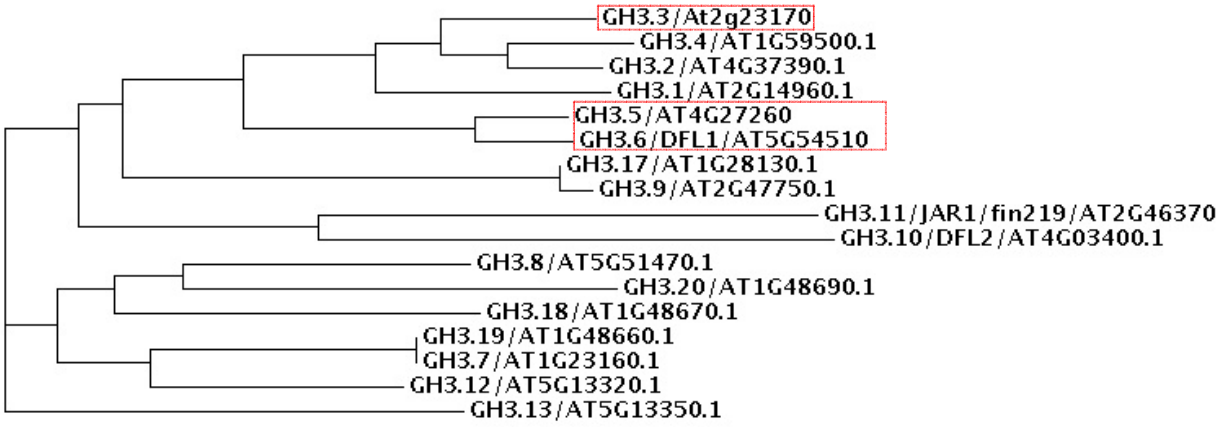


Figure 4 *Phylogenetic grouping of the GH3 gene family*. Phylogentic tree was generated using ClustalW (http://www.ebi.ac.uk/clustalw/) in which the amino acid sequence of each line was analyzed. Red boxes indicate genes positively correlated with adventitious rooting and/or auxin content (Sorin et al., 2006) that

are being assessed in this research for their potential role in the synthesis of the intermediated formed in the production of IBA from IAA.

Where group one contains the jasmonate-amido synthetase JAR1 and one other uncharacterized gene *GH3.10*, group two included genes responsible for proteins that act, at least, as IAA-amido synthetases, and group three includes genes with functions yet to be assigned (Staswick et al., 2005). Because the conjugation of IAA to amino acids is ATP-dependent, Staswick et al. (2005) was able to assess the activity of group two GH3 proteins on several auxins through monitoring the exchange of [^{32}P]-PPi into ATP. Because GH3-mediated amino acid conjugation is ATP-dependent the rate of PPi exchange is proportional to the activity of these enzymes. Intriguingly, these so-called IAA-amido synthetases were also indicated to be active on IBA. In fact, for GH3.5 it appears that IBA, not IAA, is the preferred substrate (see Figure 5).

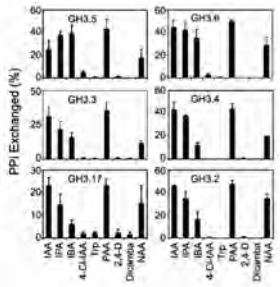


Figure 5 *Adenylation activity of GH3 proteins on several auxins* (Figure 2 in Staswick et al., 2005)

Together, the data reported by Staswick et al. (2003; 2005) and Ludwig-Müller et al. (1995a; 1995b) suggest that one of the unexplored possible routes leading to the production of product X proceeds through the GH3 catalyzed conjugation of ADP to IAA. However, the experiments conducted by Staswick et al. (2005) did not analyze the resulting conjugated products. One explanation of these results could be that GH3.5

conjugates amino acids to the carboxylic acid group on IBA more readily than IAA and does not effect the conversion step. Furthermore, these work observe the activity of these proteins on different auxins in the presence of only ATP. The structure of product X proposed by Ludwig-Müller (1995a) suggests the addition of acetyl-CoA to IAA as well. Thus, from these data present by Staswick et al. (2005) we can only assert that some GH3 proteins interact with IBA. Whether or not it is involved with its conversion of IBA from IAA remains to be conclusively determined.

As previously mentioned the exact structure of product X is unknown. Not until this compound is isolated can we begin to make reasonable hypotheses regarding the chemical alterations that might be made to IAA and extrapolate which enzymes could perhaps be involved with this step. Recent work by Park et al. (2007) demonstrate that both biotic and abiotic stress adaptation responses are mediated, at least in part, by auxin homeostasis governed through negative feedback regulation by a group two GH3 proteins (namely GH3.5, a protein of interested to this work). Lastly, it is possible that the tenuous relationship between GH3 proteins and IBA could be an artifact of the promiscuity of one or more of GH3 proteins in their ability to conjugate amino acids to indolealkanoic acids besides IAA. However, it would seem counter intuitive that this be the primary relationship between GH3 proteins and IBA. We know that *GH3* genes are induced by stress responses to sequester IAA in conjugated form, thereby inactivating the ability of IAA to function hormonally (Park et al., 2007). Furthermore, we know that IBA production increases in response to external stresses (Ludwig-Müller, 2007). From these data an intriguing question emerges: what would be the benefit of GH3-mediated amino acid conjugation of IBA under stressful conditions if such a conjugating event

would inactivate IBA from cuing these important development adaptations? An alternative explanation of the role of GH3 proteins within IAA homeostasis in response to stress that would reconcile the scenario described above would be that while some GH3 proteins function to promote IAA-conjugation events, others catalyze the conversion of IAA to IBA that exerts greater controlled over root development. Indeed, this is an intriguing hypothesis. However, there is not direct experimental evidence analyzing the enzymatic activity of each of the 17 GH3 proteins, only the 6 shown in Figure 5.

Overview of This Research

There are two primary objectives for this research. First, this research was aimed at expanding the use of GC-MS to accurately and efficiently quantify the levels of IAA and IBA in the same sample. Second, to apply this technology to address a salient biological question. The latter objective is premised on the following idea. From studies that have correlated the activity of IBA synthetase with IBA production (Ludwig-Müller 2007) it is clear that this is the primary route of IBA production within *Arabidopsis*. A mutant lacking a gene that is instrumental in the conversion of IBA to IAA in *Arabidopsis* will be characterized by an imbalanced of IBA and IAA levels, a ratio that is quantifiable via GC-MS analysis of tissue extracts of seedlings.

Putative Product X Synthase?

A recent proteomic analysis of *Arabidopsis* mutants characterized as either IAA overproducers or under producers revealed eleven proteins that positively or negatively

correlated with adventitious root development and or the formation of root primordia (Sorin et al., 2006). Three of these proteins are members of the GH3 family: GH3.3, GH3.5, and GH3.6. They were positively correlated with the formation of excessive adventitious roots in auxin over-producing mutants, suggesting that they play a specific role in the formation of these types of roots. Together with the fact that IBA induces adventitious roots to a greater degree than any other auxin (Woodward and Bartel, 2005; King and Stimart, 1998) one unexplored explanation of these findings could be that the high rate of adventitious root formation in the IAA-overproducer mutant is a result of an increased rate of GH3-catalyzed biosynthesis of IBA. This hypothesis is further supported by the idea that, in general, IBA is produced when there is an excess of IAA. Thus, the high amounts of endogenously produced IAA within this mutant could trigger the production of enzymes responsible for the conversion of IAA to IBA. This increase in IBA levels could then induce adventitious root formation. It must be noted, however, it is unknown if there exists a discrete IBA-mediated pathway, independent of IAA, that leads to the formation of adventitious roots.

As a working hypothesis, we suggest that the first of the two-step biosynthetic formation of IBA from IAA is catalyzed by an enzyme that is increasingly produced within mutants with high levels of endogenous IAA that are characterized by the formation of higher than normal numbers of adventitious roots. To test this hypothesis, lines of *Arabidopsis* lacking genes that encode the overrepresented proteins found by Sorin et al. (2006) were obtained and assayed for their ability to develop adventitious roots and other IBA-mediated events. Also, for these lines, the endogenous levels of both

IAA and IBA were to be quantified. Table 2 lists the insertional mutants used in this study.

Locus Name	Mutant Name	Arabidopsis Protein Name*	T-DNA insertion
At2g23170	*gh3-3*	GH3-3	CS104933
At4g27260	*gh3-5a*	GH3.5 / AtGH3a	SALK_033434
At4g27260	*gh3-5b*	GH3.5 / AtGH3a	SALK_151766
At5g54510	*gh3-6a*	GH3-6 / DFL1	SALK_082530
At5g54511	*gh3-6b*	GH3-6 / DFL1	SALK_140227
At5g54512	*gh3-6c*	GH3-6 / DFL1	SALK_023621
At5g54513	*gh3-6d*	GH3-6 / DFL1	SALK_060813
At3g18490	*myf24*	Aspartyl protease	SALK_045354.47.75.x
At4g38970	*At4g38970*	Putative Fru-bs-P aldolase	SALK_000898.50.00.x
At1g79930	*hsp91*	HSP91 (Heat Shock Protein)	SALK_082815.45.00.x
At1g74100	*At1g74100*	Putative flavanol sulfotransferase	SALK_003961.56.00.x
At4g25100	*fsd1*	Iron Superoxide dismutase	SALK_029455.51.70.n
At5g20630	*glp3b*	Germin-like protein GLP3b	SALK_055557.55.50.x
At1g32060	*prk*	Phosphoribulokinase	SALK_076352.55.75.x
At5g28540	*bip1*	Luminal bindind protein, putative	WiscDsLox368E04

Table 2 *Lines of* Arabidopsis thaliana *containing T-DNA insertions in genes involved in adventitious rooting or root primordial development.* The mutant names presented above denote the particular T-DNA insertion mutant used within this research. *gh3-5a, gh5-3b, gh3-6a, gh3-6b, gh3-6c, gh3-6d* represent different T-DNA insertion lines for the same gene. Protein names (*) are found at www.arabidopsis.org.

Steps to Identify a 'Product X Synthase'

The determination of the relationship between IAA and IBA as well as understanding how IBA might act as an auxin has been difficult to ascertain for numerous reasons. Despite the large body of evidence demonstrating the *in vivo* conversion of IBA from IAA, a clear biochemical mechanism for this process has yet to be established. As mentioned before, we also lack a clear understanding of any alternative biosynthetic routes IBA production may follow. Furthermore, the complete set of genes involved in the synthesis of IBA from IAA has yet to be determined. These issues stand as important gaps in our understanding of the function of IBA within plants.

This research is aimed at developing a protocol of IBA and IAA quantification that can be employed to address the key issue of identifying the enzyme(s) involved in

the production of the reaction intermediate, product X, which is converted to IBA through a reaction catalyzed by IBA synthetase. There are two basic approaches to identifying an enzyme with this activity, a genetic or biochemical approach. The former is achieved through assessing the catalytic activity of proteins purified from extracts from *in vitro* IAA to IBA biosynthesis experiments. This was the method employed by Ludwig-Müller and Hilgenberg (1995) in the identification and characterization of IBA synthetase. Work has still yet to be done to identify the gene responsible for this enzyme. The genetic approach would aim at isolating the gene or genes responsible for the enzyme involved in the reaction. These two approaches yield a different set of information. Where the identification of the enzyme allows for enzymatic activity assays that enable the measurement of IBA production under particular growth conditions and within specific tissues (Ludwig-Müller, 2007), understanding the gene(s) responsible for the production of the enzyme(s) allow for more precise characterization of the true physiological scope of IBA production within plants because it allows for important knock-out, loss of function mutational analysis of lines lacking the proteins hypothesized to be involved. Indeed, such mutant lines would be instrumental in understanding other biosynthetic routes that lead to IBA production.

GC-MS Indole-3-Butyric Acid Quantification

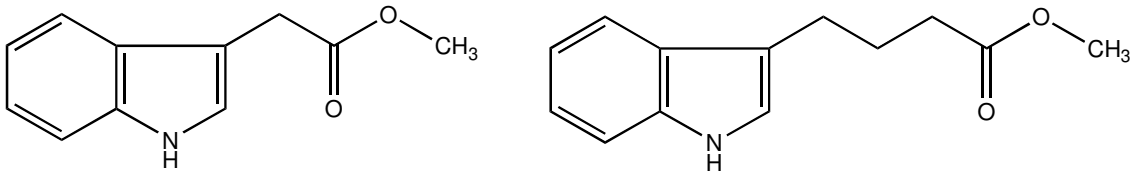
The purification and quantification of IBA and IAA has been of interest in the fields of both phytohormone biology and analytical biochemistry, as it has required insight from both fields to identify, locate, purify and analyze these specific auxins. Methods for isolation, purification, and subsequent synthesis of both IAA and IBA were

established quickly upon the identification of their major roles within plant development (see Wildman, 1997). It wasn't until the introduction of combined gas chromatography – mass spectrometry (GC-MS), however, that the correlation between the levels of endogenous auxins and selected physiological processes emerged. Anti-IAA antibodies have also been used to quantify IAA levels with a high degree of sensitivity (Mertens et al., 1985) but without analytical certainty as to what was being measured. Thus, much of what we know today about auxin activity within plants is a direct result of the quantification of these compounds with GC-MS. This technology stands as the preferred method of auxin quantification as it has demonstrated to be the most accurate and it has the ability to reveal the levels of multiple types of auxins in both free and conjugated forms (Rivier, 1986; Ilîc et al., 1996).

GC-MS quantification of IBA and IAA involves three basic steps: tissue extraction, indolealkanoic acid purification, and GC-MS analysis of the isolated compounds. During the extraction and purification steps there will be loss of product. It is therefore mandatory to correct for these losses through the addition of a known amount of a well-chosen internal standard. The preferred internal standard is an isotopomer of the compound of interest that shares its chemical properties (and thus behaves in the same manner as the native compound when subjected to the purification protocol), and can be distinctly measured upon MS analysis. The addition of an internal standard allows for an analysis that is independent of the percent of IAA or IBA lost throughout the handling and analysis of the sample. That is, because MS analysis allows for the direct quantification of compound as a ratio to the added internal standard. Thus, once the ratio of the added internal standard to plant derived hormone has been calculated, the

abundance of the endogenous auxin measured by the MS can be adjusted accordingly since the amount of internal standard added to the extract was known.

The extraction and purification of indolealkanoic acids results in free IAA and IBA in solution with some residual contaminants that can be discerned upon GC-MS analysis. Prior to GC-MS analysis, preparation of the methyl ester derivatives of these acids in which the terminal hydroxyl group is blocked upon addition of a methyl group through the exposure to diazomethane is necessary to increase the volatility of the compound (Cohen, 1984). Figure 6 gives the chemical structures of indolealkanoic acid derivatives after methylation.



methyl-indole-3-acetic acid (me-IAA) FW=189 methyl-indole-3-butyric acid (me-IBA) FW=217

Figure 6 *Chemical structures of me-IAA and me-IBA after methylation with diazomethane.*

The resulting methylated indolealkanoic acid (me-IAA or me-IBA) is then analyzed. Figure 7 highlights the fragmentation pattern of me-IAA and me-IBA upon MS analysis.

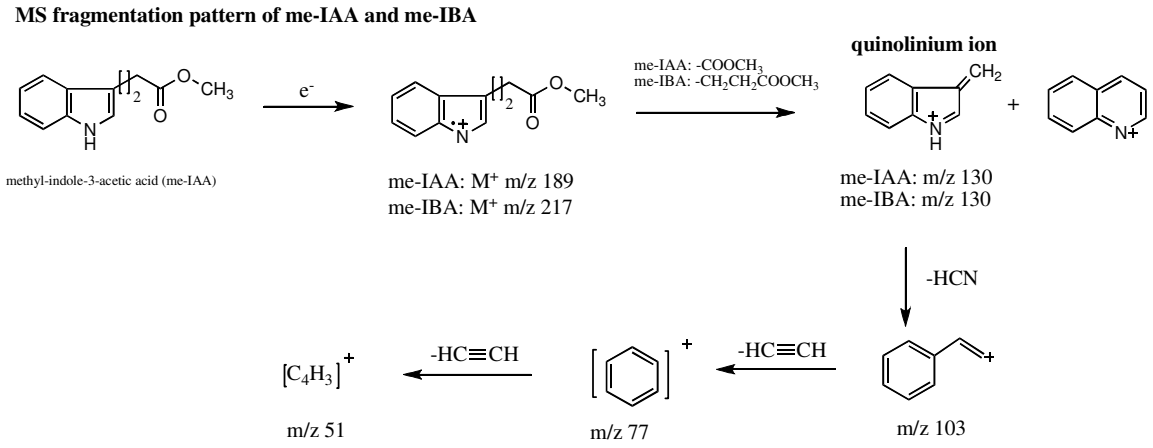


Figure 7 *Mass spectrometry fragmentation pattern of me-IAA and me-IBA.* Ions m/z 189, m/z 217 and m/z 130, respectively, are the most common ions formed. The brackets represent the two addition carbons of IBA.

This fragmentation pattern is the same with the internal standard isotopomers differing only in their respective m/z value. MS analysis highlights important characteristics of a well-designed internal standard.

The primary ions used in calculating the abundances of the IAA and IBA are the M^+ (m/z 189 and m/z 217, respectively) and the quinolinium ion (m/z 130 for both IAA and IBA). The primary ions for the internal standard will be the same fragments with m/z + *x*, where *x* is equal to the number of atomic isotopes within the internal standard. Given the integrity of the indole ring in both IAA and IBA when analyzed with GC-MS, this has been the target for isotopic enrichment of internal standards. The accuracy of the measurement is dependent upon the difference between the masses of the native compound and its isotopomer because of the natural occurrence of ^{13}C within the native compound and the incomplete enrichment of the internal standard. Thus, there will always be a calculable abundance of the primary IAA and IBA fragments, M^+ and the quinolinium ion with a m/z +1, +2, +3 etc greater than the theoretical ideal m/z of these ions. Moreover, there will also always be a small percentage of the stable isotope

internal standard that will not be fully enriched resulting primary fragments with a m/z -1, -2, -3 etc less than the assumed mass of the internal standard. Together, the need for an internal standard with the greatest mass is apparent. This would allow for the least amount of overlap between the native and internal standard fragments as read by the MS, resulting in the most precise data.

When I began this research, the highest mass (thus, preferred) stable isotope internal standard was $^{13}C_6$-[benzene ring]-indole-3-acetic acid and $^{13}C_6$-indole-3-butyric acid for the quantification of IAA and IBA, respectively, over the past twenty years (Cohen et al., 1986; Sutter and Cohen, 1992). These internal standards have been widely successful in the quantification of IAA and, to a lesser extent, IBA. This is primarily due in part to IBA existing in plants at levels that are difficult to quantify with certainty compared to IAA and the less suitable internal standard. Thus, any future work assessing endogenous levels of IBA requires a heavier internal standard.

I have capitalized on the availability of [U-$^{13}C_{8,}$ 98%+,: ^{15}N, 96-99%] indole that has made possible the synthesis of [$^{13}C_8$-^{15}N]-indole-3-butyric. This work reports the synthesis and application of this novel internal standard to quantify IBA within plants that will allow for the accurate quantification of both IAA and IBA within mutants hypothesized to be involved in the conversion of IBA from IAA.

Early research on the activity of various indolealkanoic acids suggests that indoles with longer side chains, such as indole-3-caproic acid (ICA), demonstrate auxin activity (Fawcett et al. 1960). It is thought that these auxins undergo a ß-oxidative shortening of the carboxylic acid side chain similar to that of IBA that convert them to IAA. Although indole-3-caproic acid (among other indolealkanoic acids) was shown to have auxin

activity, it has yet to be shown if this activity is a result of the promiscuous ability for the enzymes that carry out the side chain shortening pathway involved in forming IAA from IBA, or if plants synthesize indolealkanoic acids such as ICA *in vivo*. There has been little to no follow up in the past forty-five years to this simple question. If these other auxins did naturally exist, they are likely present at concentrations below the level of sensitive analytical assessment. This work also reports the synthesis of the novel internal standard [$^{13}C_8$-^{15}N]-indole-3-caproic acid that can be used in detection of ICA within plant tissue.

MATERIALS AND METHODS

Plant Material

Seeds Stock

Three different ecotypes of *Arabidopsis thaliana* were used. Colombia-0 (Col-0), Colombia-4 (Col-4), and Landsberg erecta (Ler). All insertion lines used had a Col-0 background. When unspecified, Col-0 is the assumed ecotype.

Insertion Lines

In order to create lines mutated in target genes this work relied on T-DNA insertion lines available through the Arabidopsis Biological Resource Center (ABRC) at The Ohio State University. T-DNA insertion lines were selected via the online tool SIGnAL "T-DNA Express" Arabidopsis Gene Mapping Tool (http://signal.salk.edu/cgi-bin/tdnaexpress) with which relevant genes were located (those indicated in Sorin et al., 2006) and appropriate insertion lines were selected based on the location of the insertion and the hetero/homozygosity and availability.

Growth Conditions

For the generation of seeds stocks, plants were grown in isolated pots (five plants per 4" by 4" or 2" by 2" pots) under greenhouse conditions with 16-hour light cycles and watered every other day.

Genotyping of Seed Stocks

DNA Extraction

Portions of mature leaves were collected in 1.5 ml microcentrifuge tubes and immediately transferred to ice. DNA was extracted using the REDExtract-N-Amp Plant PCR Kit protocol (Product Codes XNAPS and XNAP, Sigma-Aldrich 3050 Spruce Street, St. Louis, Missouri 63103 USA). DNA samples were stored for up to 6 months at 4˚C.

Primer and PRC Specifications

The presence or absence of the T-DNA insertion was confirmed through PRC analysis. Individual 3' primers were designed based on sequence data available through The Arabidopsis Information Resource (www.arabidopsis.org). All of the SALK insertions shared the same primer that targets the insertion DNA: TGG TTC ACG TAG TGG GCC ATC G. The WisDsLox and CS/SM insertion primer that used was: AAC GTC CGC AAT GTG TTA TTA AGT TGT C, and TAC GAA TAA GAG CGT CCA TTT TAG AGT GA, respectively. Figure A1 in the Appendix lists the genomic LP and RP of the individual insertion lines. PCR amplification was carried out using the REDExtract-N-Amp Plant PCR protocol with the following adjustments in order to maximize the utility of each kit. Total volume of 'master mix' was reduced from 20µl to 12µl by reducing the Ready-Mix volume to 6µl and the DNA solution to 1µl. Final concentration of LP and RP primers was 0.5µM. The volume of the remaining reagents was adjusted accordingly. PCR cycling parameters were as follows: 94˚C 3mn; 35-45 cycles of 94˚C 1mn, 57˚C 0.5mn, 72˚C 2mn; 72˚C 10mn; cooled to 4˚C. PCR products

were separated and visualized using 1.2% agarose with 0.005% ethidium bromide gel electrophoresis run for 45mn at 90V and visualized with a Alpha Innotech Coporation camera and UV source (Model #2.1.1, San Leandro, CA) and Alpha Ease FluoroChem 5500 software. Figure A2 in the Appendix shows the gels used to identify the homozygous progeny used in this study. Seeds from insertion lines SALK_033434 and CS104933 used in this study were grown from homozygous seed stocks that were directly order from T-DNA Express.

Growth Conditions

Seed Sterilization

All phenotypic assays and seedlings used for IAA and IBA quantification were grown under sterile conditions. Prior to sowing the seeds in growth media, 20-200 seeds were collected in a 1.5 ml microcentrifuge tube for surface sterilization which was done by adding 800µl 95% ethyl alcohol, mixed with vortexing, and the solution was aspirated off with pipette within 1 minute of application, followed by the addition of 800 µl of a 20% bleach 0.05% Tween-20 solution, mixed with vortexing, and the solution again was aspirated off, followed by two rinses with 800µl dH_2O. Seeds were either plated directly or stored up to a week at 4˚C.

Growth Media

Growth media contained 0.7% Cassion phytablend agar, 0.44% Musrashige and Skog salts with macronutrients and vitamins (MSP002), pH 5.6 (adjusted with 0.1 KOH). Liquid media contained the above ingredients without the agar. Media was autoclaved,

cooled, and poured into sterile Petri dishes (150x15mm) in a laminar flow hood and allowed to cool. Sterilized seeds were sown or 'plated' onto Petri dishes in a laminar flow hood and immediate wrapped with Parafilm. Plates were placed under the growth conditions specified by the experiment.

Experimental Growth Conditions for Indolealkanoic Quantification

For IAA and IBA quantification experiments surfaced sterilized seeds were grown in either liquid or solid growth media in a growth chamber under continuous light (140-160 µmol) at 24˚C. Liquid cultures were shaken at 100-150 rpm.

Adventitious Root Assay

Adventitious rooting assays were carried out as follows. Seeds were prepared and plated in 2 to 4 parallel rows on the media and placed in a light proof canister (Fisher Brand autoclave canister) sealed with light-proof tape and placed in the growth chamber so that the rows were perpendicular to the gravity vector at 4˚C for 18-48 hours, unwrapped and exposed to light (~140-160 µmol) for 1 hour, placed back in the light-tight canister and placed vertically in a growth chamber at 24˚C for 5 days (etiolation period). After the etiolation period, plates were placed vertically in a growth chamber and subjected to either a 16/8 hour light/dark cycle or continuous light for 10 to 15 days. The adventitious were counted, or 'scored,' with the aid of a dissecting microscope.

Hypocotyl and Root Elongation Assays

Hypocotyl elongation assays followed the same steps as for the adventitious rooting assay. However, hypocotyl lengths were measured directly after the etiolation period. Root elongation experiments were carried out by placing the plated vertically in a continuous light source at 24˚C and scored with 10 to 14 days. Photographs of etiolated seedlings and vertically grown seedlings were taken with an Alpha Innotech Coporation light source and camera (Model #2.1.1, San Leandro, CA) and visualized with Alpha Ease FluoroChem 5500 software. ImageJ software (available at: http://rsb.info.nih.gov/ij/) was used to make measurements through calibration of pixel distance based on a ruler placed on the media within every picture.

Synthesis of Indolealkanoic Internal Standards

Synthesis of [$^{13}C_8$-^{15}N]-Indole-3-Butyric Acid

The synthesis and purification of [$^{13}C_8$-^{15}N]-IBA and [$^{13}C_8$-^{15}N]-ICA followed the basic steps of indolealkanoic acid synthesis as described by Cohen et al. (1986). This reaction follows two basic steps, the opening of the lactone ring and its subsequent addition to the C3 of the indole ring (See Figure 7):

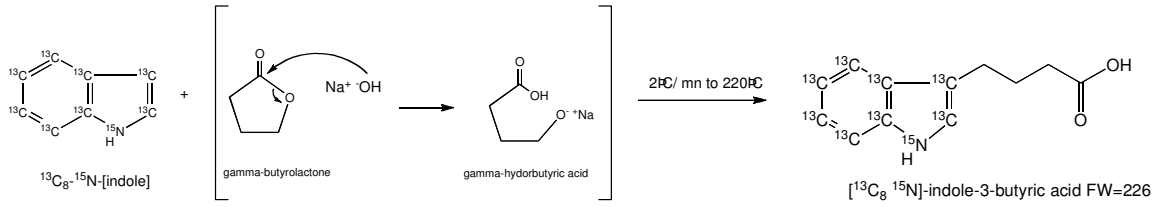


Figure 7 *IBA is the product of a two step process of the opening of a lactone ring and subsequent attack on the C3 of the indole ring*. The synthesis of ICA follows a similar path with the substitution of gamma butyrolactone with 6-caprolactone.

For the synthesis of [$^{13}C_8$-^{15}N]-IBA 0.08 moles of gamma butyrolactone, 0.08 moles NaOH, and $4.26x10^{-4}$ moles (0.05g) of [U-13C8, 98%+;15N, 96-99%]-indole (Lot# PR-11049, Catalog # CNLM-4786-0, Cambridge Isotope Laboratories Inc., Andover, MA) were mixed together in a 23 mL Teflon insert (Catalog # A280-AC, Parr Instruments, Moline Illinois) and fitted into a screw-top reaction bomb (Catalog# 276AC-T304-012304, Parr Instruments, Moline Illinois). The reaction bomb was heated to 220˚C at a rate of 2˚C/minute in a sand bath and incubated at 220˚C for 24 hours. The reaction mixture (consisting of a brown liquid layer and solid white layer) was removed from the Teflon insert and placed in an Erlenmeyer flask. 50 ml of dH_2O was added to stop the reaction. The mixture was stirred until the solid white mass was completely dissolved.

Purification of [$^{13}C_8$-^{15}N]-IBA from Reaction Products

The synthesized indolealkanoic acid internal standard was purified from the reaction mixture by the following partitioning sequence. The aqueous reaction mixture and 125 ml chloroform (density = 1.412) was added to a separatory funnel, mixed thoroughly and allowed to phase separate. The chloroform layer was removed and another 125 ml of chloroform is added and collected. The chloroform layer contains any unreacted indole. After the chloroform layer had been removed the aqueous layer was collected and brought to pH 2.5 with 6N HCl and added to a new separatory funnel with an equal volume of ethyl acetate (density = 0.9). This step was repeated three times, partitioning off and discarding the aqueous layer each time. The ethyl acetate layer

containing the product was transferred to a round bottom flask and evaporated using a rotovap and redissolved in 12 ml 50% isopropanol for storage.

Due to the expense of the labeled indole and the low percent yield of this reaction, the unreacted starting material was recovered by evaporating the chloroform layer with a rotovap and redissolved in ~20 ml dichloromethane for later use. The presence of the indole was confirmed via TLC (see *Thin Layer Chromatography* section of Materials and Methods) and GC-SIM-MS, and purified via liquid phase chromatography as described below (see *Synthesis of [$^{13}C_8$-^{15}N]-Indole-3-Caproic Acid* section of Materials and Methods). Figure A3 in the Appendix displays the GC-MS spectra of the recovered indole.

Product Yield Calculation

The product yield was calculated via gas chromatography-selected ion monitoring-mass spectrometry (GC-SIM-MS). To measure the amount of [$^{13}C_8$-^{15}N]-IBA synthesized, what was estimated to be equal amounts of the IBA internal standard and an accurate determined amount of unlabeled IBA (for this a 5mg/50 ml IBA/methanol solution was prepared) were methylated and analyzed with GC-SIM-MS.. To determine the concentration of the IBA internal standard, and estimation of the final concentration was made in order to add approximately an equal amounts of the IBA internal standard and IBA together for GC-SIM-MS analysis. 2 µl (1µg) of the 5mg/50 ml IBA/methanol solution was added and 5 µl of the product in 50% isopropanol were added together in a GC-SIM-MS vial, dried over N_2 and methylated with an ethereal solution of diazomethane (see Cohen, 1984), and resuspended in 15µl ethyl acetate for

GC-SIM-MS analysis. Ions at m/z 139 and 226 were monitored for the base peak (quinolinium ion) and molecular ion, respectively, of the [$^{13}C_8$-^{15}N]-IBA internal standard and ions at m/z 130 and 217, the base peak and the molecular ion of IBA were also monitored. The ratio of 130:139 was used to calculate the amount of [$^{13}C_8$-^{15}N]-IBA synthesized and the ratio of 217:226 was used for confirmation. This procedure was preferred over an average of the two ratios because of the better ion statistics for the more abundant base peak (Cohen et al., 1984).

Synthesis of [$^{13}C_8$-^{15}N]-Indole-3-Caproic Acid

The synthesis and purification of [$^{13}C_8$-^{15}N]-indole-3-caproic ([$^{13}C_8$-^{15}N]-ICA) followed the basic guidelines outlined above. However, the [$^{13}C_8$-^{15}N]-indole used in this reaction was recovered from the incomplete synthesis of [$^{13}C_8$-^{15}N]-IBA described above. After the chloroform layer was evaporated and the unreacted products are redissolved in dichloromethane, the presence of the indole was confirmed via TLC. The purpose of the chloroform partition step was to crudely separate out the nonpolar contaminates and the unreacted indole from the polar indolealkanoic acid that is soluble in the aqueous layer.

Reverse-phase liquid chromatography with a polar to nonpolar elution gradient was used to purify the unreacted indole from the other unknown contaminants present in the chloroform layer. The unreacted products that were dissolved in dichloromethane were dried over N_2 and redissolved in 2-3 ml 50% isopropanol. A 1 x 50 cm column was filled with Sephadex LH-20 (in 50% isopropanol) and connected to an automated fraction collector set to collect 250 drops (~3 ml). The sample was applied to the column. Once

the sample was applied to the lipophilic Sephadex column, a 50% to 100% isopropanol gradient was drawn into the closed system (see Figure 9).

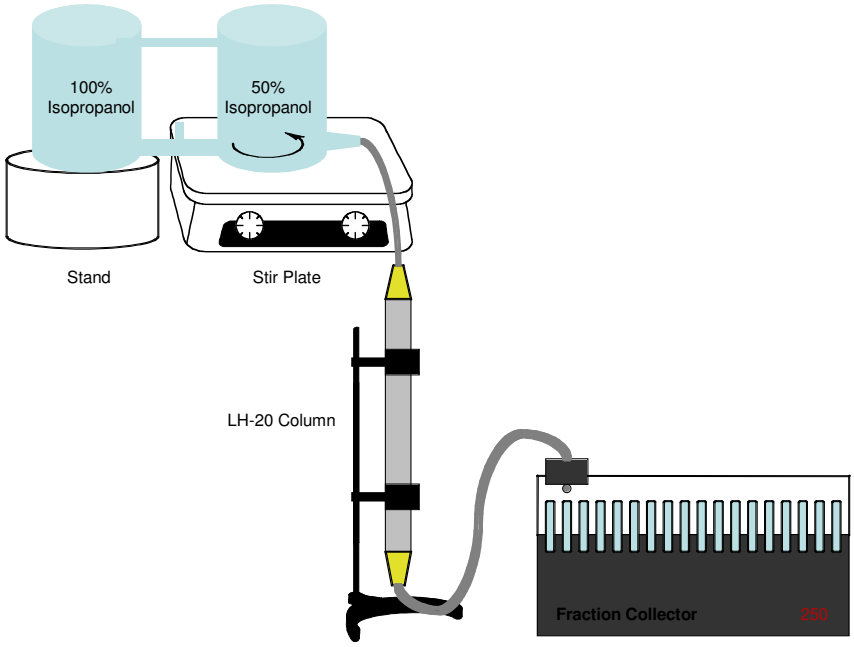


Figure 9 *The gradient elution system for the purification of indole via reverse-phase liquid chromatography*. The 50% to 100% isopropanol gradient is generated as the column draws eluent from the gradient maker that will becoming increasingly polar. The system is connected by tubing and driven by gravity.

As the eluent was drawn through the system and fractions collected, the newly added solvent becomes increasingly polar. Thus, compounds are separated and eluted based on their decreasing polarity. Fractions of 250 drops are collected for 10-24 hours. 5 µl from each fraction was spotted onto a silica gel 60 F_{254} plate and stained directly for the presence of indole (see *Thin Layer Chromatography* in Materials and Methods). Fractions that stained positive for indole were combined and the presence of indole confirmed via GC-SIM-MS by taking a 5µl sample of the collected fractions, drying with a stream of N_2 and the residue redissolved in ethyl acetate and then the sample was analyzed by GC-SIM-MS.

The goal of using GC-SIM-MS was two fold: to verify the presence of the labeled indole and to calculate the percent enrichment of the indole. This was calculated with the data in Figure A3 in the Appendix where the retention time of 6.2 to 6.4 minutes of indole was verified by a previously run standard and the m/z 126 represented the molecular ion of the fully enriched [$^{13}C_8$-^{15}N]-indole, and m/z 117 the molecular ion of unlabeled indole.

Thin Layer Chromatography

Indole and Indolealkanoic acids were visualized via thin layer chromatography (TLC) under the following conditions. Samples were spotted onto a standard silica gel 60 F_{254}. The developing solvent was 85:14:1 chloroform:methanol:dH_2O solution. The staining solution for both indolealkanoic acids and indole used was Ehmann's reagent (Ehmann A, 1977). Ehmann reagent is a 3:1 mixture of Salkowski's reagent and Ehrlich's reagent. 800 ml of Salkowski's reagent was prepared on ice by combing 2.06 g hydro-$FeCl_3$, 500 ml dH_2O, 300 ml concentrated sulfuric acid, pouring the acid into water very slowly (extremely exothermic reaction). 100 ml of Elrlich's reagent was prepared by combining 1 g *p*-dimethylaminobenzaldehyde, 50 ml concentrated HCl, and 50 ml absolute ethanol.

The TCL plate was placed vertically in a jar where the atmosphere was saturated with solvent vapor and containing enough solvent to cover the bottom to a depth of about 1 cm. After development in the solvent the plate was removed, residual solvent allowed to evaporate and the plate is submerged into Ehamm's reagent (freshly prepared) for 5 minutes, removed, blotted dry with a paper towel and placed at 100˚C for 5 minutes. The

plate was then fixed permanently by submerging it into a circulating water bath for 30 minutes.

High Throughput Quantification of IBA and IAA

The quantification of both IAA and IBA followed the general protocol reported by Barkawi et al. (in preparation). The primary refinement presented here is a post-SPE purification step of methylated samples from which IBA and IAA measurements were calculated from a single biological sample.

Tissue collection

Seedlings were grown under the specified experimental conditions. Samples were be collected, weighed, and were either directly extracted or placed at -80˚C for storage until use.

For both liquid and solid media cultures, Whatman filter paper was placed in the bottom of a Buchner funnel to which a vacuum was applied. The tissue was collected into the funnel and washed thoroughly with dH_2O and dried with the running vacuum for 5 to 10 minutes.

Eppendorf tubes were labeled and their masses were determined. The dried sample was added to the tube. The mass of the tube containing the dried tissue sample was determined. Measurements to the ten-thousandths of a gram were taken. Samples were immediately place in either an ethanol-dry ice bath or liquid nitrogen for transportation and placed at -80˚C for storage.

Pipettes and Tips

In order eliminate phthalate contamination within samples analyzed using GC-MS. Gilson Microman positive-displacement pipettes together with disposable capillary piston (Gilson, Middletown, Wisconsin) tips were used in every experiment in which samples were to be eventually analyzed with the GC-MS.

Determining internal standard concentrations with Ultra Violet Spectrometry

Ultra violet spectrophotometry (UV spectrophotometry) was used to determine the concentration of internal standard added to each sample. An average of three measurements were taken with a Hewlett Packard 8453 UV Spectrophotometry using a 10.00 mm QS 50µl quartz cuvette (Agilent, Germany). Data was computed with 845x UV-Visible ChemStation System 1 software and averaged for the final concentration measurements. Indole-containing compounds have a maximal UV absorbance at 282 nm with an extinction coefficient of 6060 cm^{-1}•$(moles/liter)^{-1}$. Concentrations of indole containing compounds can be calculated with Beer's Law:

$$A_{282} = \varepsilon c l$$

where A_{282} is the absorbance at wavelength 282 nm, ε is the extinction coefficient, c is the concentration (molarity) and l is the length of the light path for the cuvette in centimeters.

Tissue Preparation and Disruption/Extraction

Tissue homogenization was performed in individual 1.5 ml Eppendorf tubes. To each tube 150 µl homogenization buffer (65% isopropanol, 35% 0.2M imidizole, pH 7), 1-3 tungsten carbide beads (Qiagen, Valencia, California) were added along with a known amount of the appropriate internal standard. Approximately 40ng/g fresh weight and 20ng/g fresh weight of IAA and IBA internal standards was added, respectively.

Samples containing the tissue, metal beads, internal standards, and homogenization buffer are homogenized using a Mixer Mill MM 300 (Qiagen) at 15 Hz for 4 minutes. Homogenates are equilibrated on ice for 1 hour, centrifuged for 5 minutes at 10,000 x *g*. The supernatants of the sample were transferred with Pasteur pipettes to a deep 96-well microplate on the sample rack of an automated liquid handler (ALH).

ALH Sequence

An eight probe 215 SPE automated liquid handler (Gilson, Middleton, Wisconsin; see Figure 10) was programmed to carry our the sample purification of indolealkaonic acids from tissue homogenate by solid phase extraction, using positive pressure, provided from a nitrogen gas line (ultra-high purity [UHP], <0.5 ppm oxygen), to carry out elution steps. The ALH was equipped with the following Gilson racks: 1 Code 205H rack to accommodate two deep-well 96 well microplates; 2 custom racks to hold Varian Versaplate solid phase extraction tubes (Varian, Lake Forest, California); and 2 Code 228 racks that each hold four 700 ml reagent bottles (solvent and eluents, Figure 10). Liquid transfers and positive pressure elutions are carried out with eight 1 ml syringes. The ALH was controlled using Gilson's 735 Instrument Control V 5.10 Software.

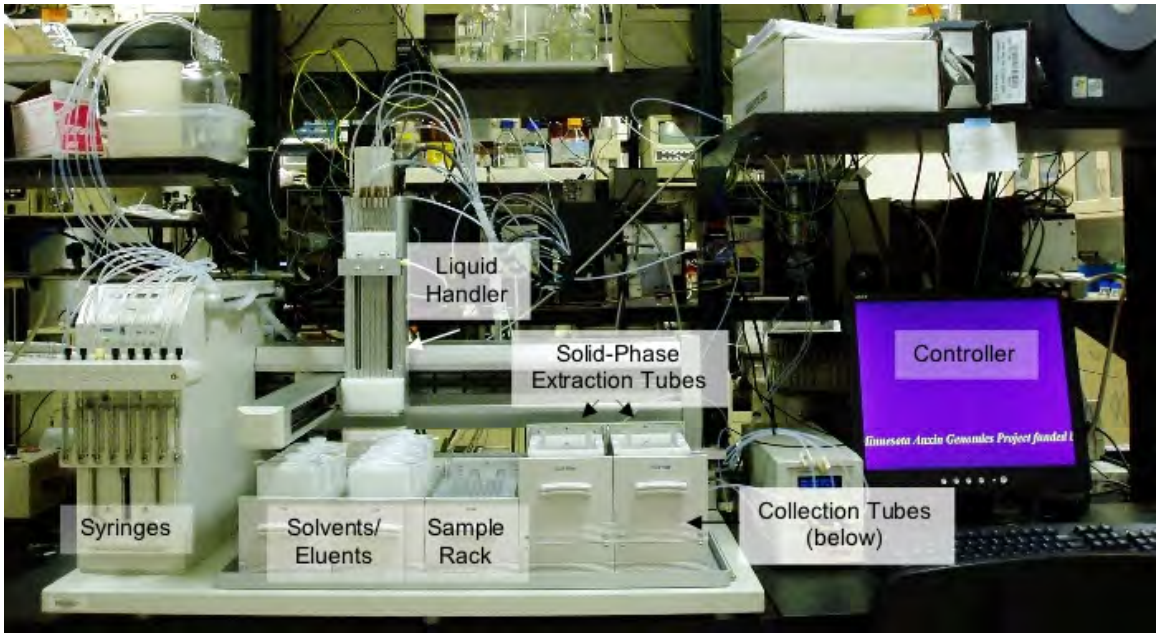


Figure 10 *Automated Liquid Handler.* Homogenated samples are placed on the sample rack. The liquid handler transfers the solvents and eluents to solid-phase extraction tubes in sequence.

Solid Phase Extraction (SPE)

Once loaded onto sample rack, homogenized samples are extracted using the following protocol that was programmed into the ALH control software. For the protocol nitrogen pressure was set at 20 psi and each step in the method that called for positive pressure to push liquid through columns flow rate was set at 6 ml/min. Amino anion exchange columns (50 mg; Varian) were sequentially conditioned with 0.6 ml of hexane, acetonitrile, water and 0.2M imidazole, followed by 3 x 1.2 ml water. Samples were diluted to 10x with 1.35 ml dH_2O, mixed thoroughly, and loaded onto the columns and the column was dried with nitrogen for 5 minutes. After which, the samples were pushed through the columns and the column was dried with nitrogen for 5 minutes. The columns were sequentially rinsed for on-column clean-up with 0.6 ml each of hexane, ethyl acetate, acetonitrile, and methanol. The SPE rack was programmed to move over a deep-

well collection plate, and IAA and IBA was eluted form the columns using 1.8 ml of 0.25% phosphoric acid. To raise the pH of the eluate to 3.5, 380 µl of a 0.1 M succinic acid was added and mixed thoroughly. The second SPE rack contained SPE tubes that had been prepped with 1 ml of a 25% (w/v) suspension of polymethylmethacrylate (PMME) resin (Affi-Prep, BioRad, Herclues, California) in 0.1 $NaHCO_3$ pH 7 (which results in columns that contain about 200 mg of resin). The residual loading liquid was pushed through, leaving the resin in the tubes, which were then conditioned to pH 3.5 using 3 x 0.6 ml 6:1 phosphoric acid: succinic acid (v/v). The pH-adjusted eluate was then loaded onto the PMME columns, and after a 5 minute wait time, was pushed through the columns. The columns were then rinsed using 3 x 0.6 ml 6:1 phosphoric acid: succinic acid (v/v) followed by 300 µl methanol. The SPE rack was moved over a collection plate and eluted with a second 300 µl methanol. The first volume of methanol served to dry the column with minimum loss of IAA and IBA and the second volume of methanol was the sample eluent, containing the larger percentage of IAA.

Methylation of Samples

Samples were transferred to GS-MS glass vials with Pasteur pipettes. Approximately 1-2 ml of ethereal diazomethane was carefully added to the vials with a Pasteur pipette. Diazomethane is an extremely dangerous explosive chemical that can cause serious respiratory distress and can cause damage to tissues and should be handle with great care minimizing exposure by use of a well vented solvent hood and limiting the amount of time of use. Samples were allowed to equilibrate for 5 minutes and dried over nitrogen in a heated sand bath. Samples analyzed solely for IAA were redissolved

in 15 µl ethyl acetate and transferred to new GC-MS vials with a 50 µl glass insert to accommodate the small volume of sample for direct GC-SIM-MS analysis. Samples analyzed for both IBA and IAA were redissolved in 50 µl 0.2 M imidazole pH 7 for post-SPE indolealkanoic acid purification.

Post-SPE Indolealkanoic Purification

A 96-column holder fitted to a vacuum source was used as the negative pressure system to draw through eluents of the post-SPE purification. To maximize the contact time with the column the samples and elutions were pulled through the columns with a vacuum pressure between -10 and -20 kPa. C18 columns (Varian) were conditioned with 2 x 250 µl 100% methanol followed by 2 x 250 µl water. Samples dissolved in 50 µl 0.2 M imidazole pH 7 were loaded to conditioned columns, allowed to equilibrate for 5 minutes and pulled through slowly. Columns were washed sequentially with 250µl of 10%, 20%, and 30% methanol solutions, discarding the washes. Columns were eluted with 3 x 250 µl of 60% methanol, collecting each elution in a deep-well 96 well plate for a total volume of 750 µl. Samples then were transferred to a glass GC-MS vial with a Pasteur pipette, dried over nitrogen in a heated sand bath and redissolved in 15 µl ethyl acetate and transferred to new GC-MS vials with a 50 µl glass insert. Vials were capped and samples analyzed via GC-SIM-MS.

GC-SIM-MS Analysis

Two GC-SIM-MS instruments were used in this research. GC-SIM-SM analysis of all tissue samples were carried out with a combined 6890 Series CG System – 5973

Network Mass Spectrometry Detector (Agilent) with the appropriate software under the following specifications: HP19091S-433 capillary inlet system with HP-5MS 5% Phenyl Methyl Siloxane column with a film thickness of 0.25 µm, nominal diameter 25.00 µm. SIM analysis for IAA and IBA was done using a 7 ion program dwelling on each ion for 50 ms. For methyl-IAA using [$^{13}C_6$]-IAA as an internal standard, the ions m/z 130, 136, 189, and 195 were monitored. For methyl-IBA using [$^{13}C_8$-^{15}N]-IBA as the internal standard, the ions m/z 130, 139, 217, and 226 were monitored. The injector was heated at 280˚C and He at 50.0 ml/mn (purge rate) and 20.0 ml/mn (saver rate) with a pressure of 8.81 psi was the carrier gas. With the HP oven at 70˚C for 2 minutes followed by temperature programming at 20˚C/mn for 5 mn with a final temperature of 280˚C. The columns were held at 280˚C for 0.5 mn. Injections were made in the splitless mode through the column at a constant flow rate of 1 ml/mn with an average velocity of 37 cm/sec with a total run time of 17.5 mn for each sample. An ethyl acetate blank was used between each sample.

The amount of free IAA and IBA in samples was calculated from tabulated data from each spectrum using a modification of the isotope dilution equation presented above (see *Quantification of IAA and IBA in* Arabidopsis thaliana in Results and Discussion). Ions at m/z 136 and 195 were monitored for the base peak and molecular ion, respectively, of the [$^{13}C_6$]-IAA internal standard and ions at m/z 130 and 189 for the base peak and molecular ion of endogenous IAA. Ions at m/z 139 and 226 were monitored for the base peak (quinolinium ion) and molecular ion, respectively, of the [$^{13}C_8$-^{15}N]-IBA internal standard and ions at m/z 130 and 217, the base peak and the molecular ion of IBA were also monitored. The ratio of 130:139 was used to calculate the amount of free

IBA present in the sample and the ratio of 217:226 was used for confirmation. The ratio of 130:136 was used to calculate the amount of free IAA in the sample and the ratio of 189:195 was used for confirmation.

GC-SIM-MS analysis of the isolated labeled indole and the IBA internal standard for *R* value calculations were generated with a HP 5890A gas chromatography machine and HP 5970 mass selected detector. This GS-SIM-MS machine is calibrated somewhat differently than the GS-SIM-MS used in IAA/IBA quantification and is used when low level trace analysis is not required. For our purposes, the major different between these machines is the retention time of IBA on the HP5890A GC-SIM-MS (~10.2 mn) is slightly less than that of the first machine (~11.4 mn).

Refining Post-SPE Purification of Samples

Radioactive Labeling Experiments

The elution pattern of IBA for the post-SPE purification was determined by tracking the elution pattern of methylated [^{3}H]-IBA (Lot# 020814 ART 1112-IBA[3H], American Radiolabeled Chemicals, St. Louis, MO). Radioactivity was measured with a Beckman-Coulter LS6500 Multi-Purpose Scintillation Counter (Fullerton, California) measuring 3H radioactivity at dpm/mn for 1 minute for each sample with quench limits of 7.174 to 329.

Methylation of [^{3}H]-IBA

10 µl of a 1000 dpm solution of [^{3}H]-IBA was added to each sample after SPE. Ethereal diazomethane was added to each sample as describe above, dried over nitrogen and dissolved in 20 µl ethyl acetate. 5 µl of this solution was spotted onto a TLC plate with me-IBA and IBA standards. 5 mm sections of the silica gel were cut using a razor blade and collected into 5 ml scintillation tube to which 4 ml of a biodegradable counting cocktail (Research Products International, Mount Prospect Ill.) was added. Samples were capped and counted on a scintillation counter.

Determining the Elution Pattern of [^{3}H]-IBA

[^{3}H]-IBA was added to samples after SPE, methylated and were added to C18 columns prepared as described above. The initial flow through (after 5 minutes equilibration) and the following series of elutions were collected into 5ml scintillation vials: 250µl of 10%, 20%, 30%, and 50% methanol solutions. The resin within the C18 column was also collected. Radioactivity was measured as described above. Two independent trails of this experiment were run and the data was calculated by comparing the percent of each vial with the total amount of radioactivity measured within the sample vials.

Expanding the IAA-Specific Clean Up Protocol

[^{3}H]-IBA was added to samples after SPE, methylated and were added to C18 columns prepared as described above. The initial flow through (after 5 minutes equilibration) and the following series of elutions were collected into 5ml scintillation

vials: 250µl of 10%, 20%, 30%, 40%, 45%, 50%, 55%, 60%, 65%, and 75% methanol solutions. The flow through and each elution and the final C18 column material were collected into scintillation vials and radioactivity was measured.

RESULTS AND DISCUSSION

Phenotypic Growth Assays

The goal of the phenotypic assays was to determine the effects of the loss of genes encoding the proteins that were correlated with adventitious root formation and/or endogenous IAA content. Sorin et al. (2006) identified 11 proteins that they predicted to be involved in different biological processes including the regulation of auxin homeostasis and light-associated metabolic pathways. Auxin homeostasis is carried out by mechanisms that help maintain a suitable level of IAA in response to external stimulus and developmental cues. IAA levels can be altered through the conjugation of IAA to amino acids as well as the conversion of IAA to IBA. Because the proteins that were identified by Sorin et al. (2006) were broadly defined as potentially involved in auxin homeostasis as well as other biological processes it was important for this work to figure out which proteins play a role in auxin-mediated processes involve, more specifically, if any of these auxin-mediated processes demand the production of IBA from IAA. I performed three sets of assays to evaluate three auxin-mediated developmental processes: adventitious root formation, hypocotyls elongation, and root elongation.

Adventitious Rooting Assay

Adventitious rooting is a developmental event heavily influenced by auxin, especially IAA (Woodward and Bartel, 2005; King and Stimart, 1998). The ability of each mutant line was assayed for their ability to form adventitious roots. Figure 11 shows the average number of adventitious roots formed per seedling of the mutant line.

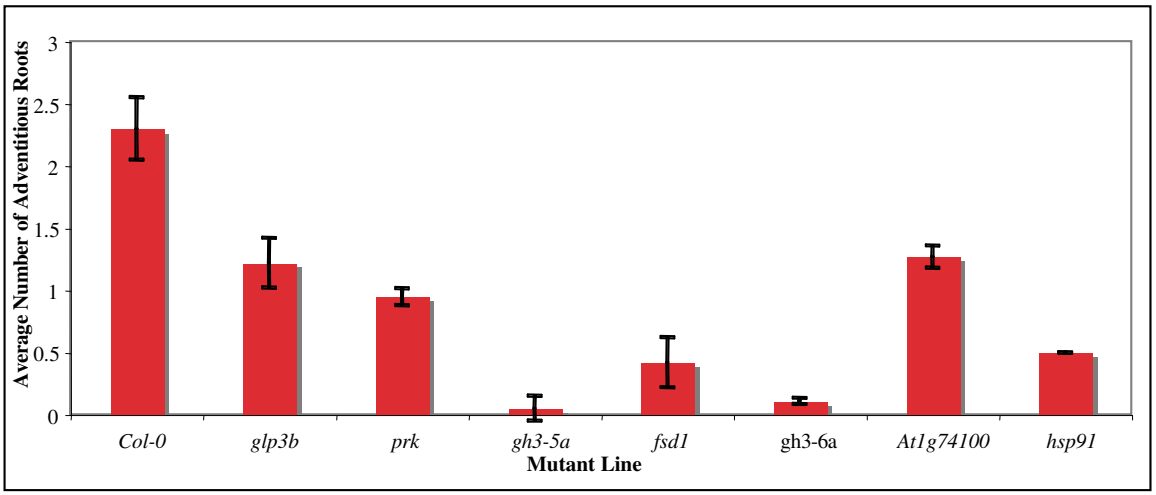


Figure 11 *Average number of adventitious roots in insertion lines are less than Col-0.* Seeds were surfaced sterilized and plated on GM held at 4˚C for 24 hours, exposed to light for 8 hours, etiolated in complete darkness for 5 days and scored after 18 days of germination. Error bar represent the standard deviation of the number of adventitious roots among the seedlings (n=40-80).

Notably, each of the insertion lines have an impaired ability to form adventitious roots under these experimental conditions. The mutant *gh3-5a*, *fsd1*, and *gh3-6a* have the most impaired ability to form adventitious roots compared to all others, implying that these proteins potentially play a role in auxin homeostasis. The *gh3-5a* mutant displayed virtually no adventitious roots under these experimental conditions.

These results are in line with the findings of Sorin et al. (2006). They found that GLP3, GH3-5, AT1G74100, and HSP91 production correlated with adventitious rooting. Thus, in lines in which these proteins are not expressed it is to be expected that these plants will demonstrate an impaired ability to form adventitious roots.

Hypocotyl and Root Elongation Assays

Both IAA and IBA are known to be involved in the elongation of the hypocotyls of *Arabidopsis* seedlings (Rashotte et al. 2003; Gray et al., 1998). Furthermore, root elongation is highly dependent upon auxin homeostasis (Celenza et al. 1995). Both of

these assays can be used to assess auxin homeostasis with *Arabidopsis* seedlings. Figures 12 and 13 display the results from these assays.

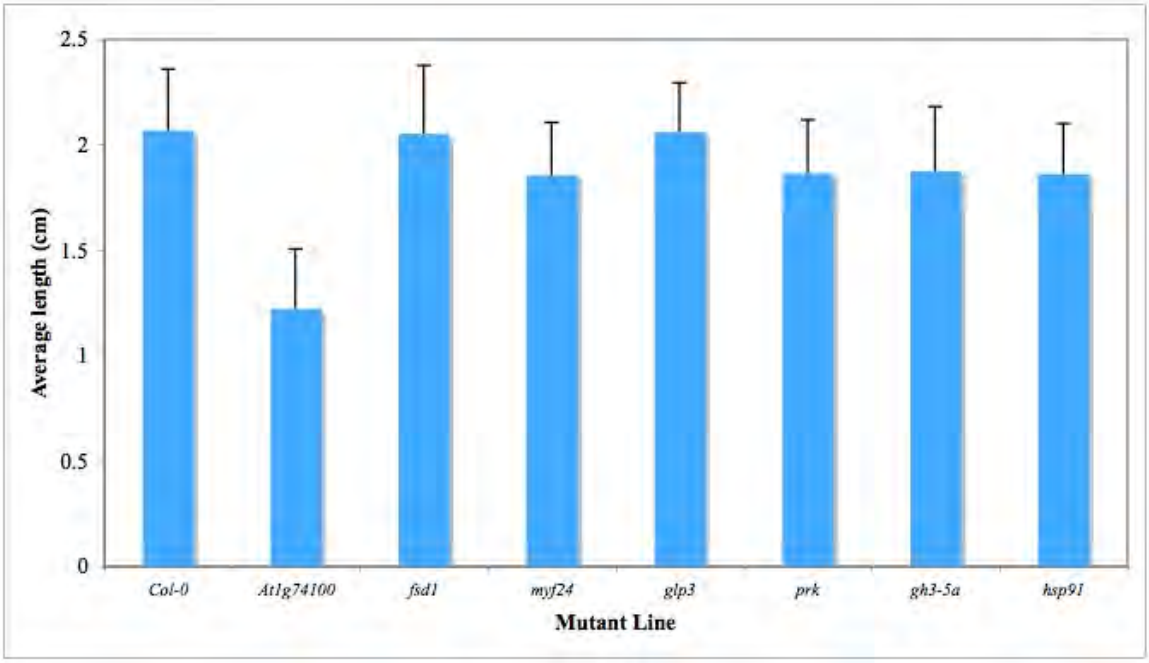


Figure 12 *Hypocotyl length of 7 day-old* Arabidopsis *insertion mutant lines.* Seedlings were grown vertically with respect to the gravity vector on GM. Seedlings were sown, placed at 4˚C for 24 hours, exposed to light for 8 hours at 24˚C for 8 hours, etiolated in complete darkness for 5 days and exposed to light for 7 days. Error bars represent the standard deviations within each population of seedlings (n=50-80).

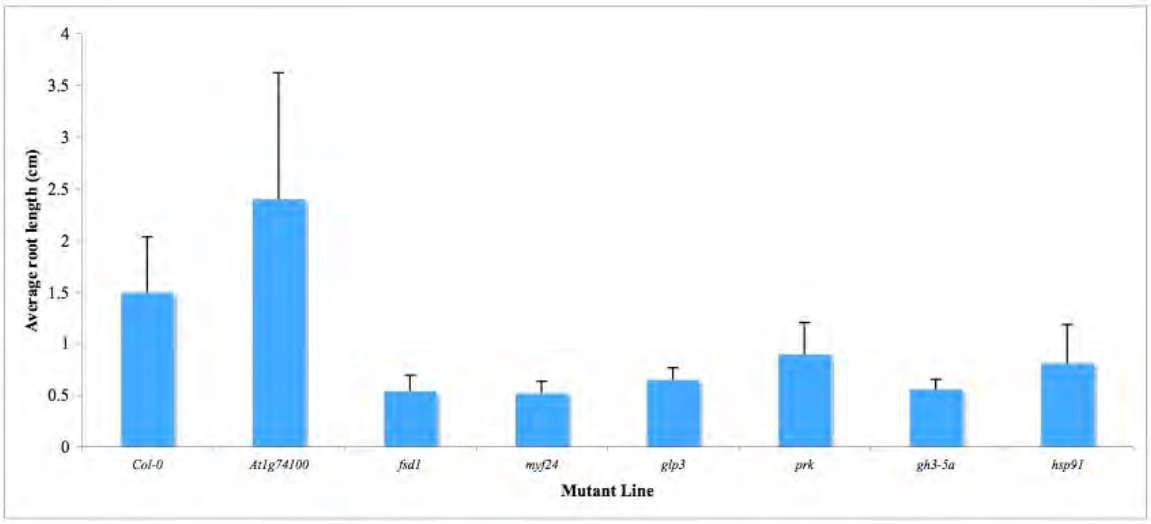


Figure 13 *Root length of 9 day-old* Arabidopsis *insertion mutant lines.* Seedlings were grown vertically with respect to the gravity vector on GM for 9 days under continuous light at 24˚C. Error bars represent the standard deviations within each population of seedlings (n=50-75).

When growth media is supplemented with synthetically produced IBA two very distinct effects results: decreased primary root elongation and increased lateral root formation. By extension, high levels of endogenous IBA should demonstrate similar effects. However, it should be noted that there have been no published reports correlating these phenotypes with IBA over- and under-producers. In the context of this study, an insertion line with an aberrant IBA biosynthetic pathway would be expected to have uncharacteristically long roots when grown under the conditions described above.

Interestingly, the *mfy24* mutant (MFY24 has identified as an aspartyl protease and a putative chloroplast nucleiod DNA binding protein[Sorin et al. 2006]) has an apparent impairment in hypocotyl elongation and an increase ability of root elongation. It is difficult to say with any certainty why this aspartyl protease mutant demonstrates increased root growth and decrease hypocotyls elongation without further analysis of this protein. However, it is unlikely that this protein plays a direct role in the conversion of metabolic products of IAA homeostasis (see Figure 2).

I measured the relative concentrations of IAA and IBA within seedlings grown under these conditions in order to firmly establish the relationship between root elongation and IBA concentrations within these lines. It should be kept in mind, however, that there are many other factors that are involved in root elongation including other hormonal relationship such as the involvement of the phytohormone gibberellin (Fu and Harber et al., 2003), or the availability of nutrients such as nitrates (Zhang et al., 1998)

Together, these data revealed a general trend for mutant lines to be impaired in biological processes that are dependent upon auxin homeostasis. However, they also

provide limited insight of the role IBA within each of these processes. From these data we cannot say conclusively that IBA is directly responsible for these observed phenotypes. When comparing the adventitious rooting of these mutants to their root elongation data it becomes difficult to theorize a specific pattern of hormone control, especially in terms of IBA regulation and production.

Although phenotypic data of mutants cannot provide conclusive insights to the mechanism of protein function is provides the necessary biological data to generally contextualize the potential role of the encoded protein. These results demonstrate the potential importance of each of these proteins within auxin homeostasis system and therefore point to the importance of more direct analysis. This research was concerned with pinpointing the enzyme involved in the interconversion of IAA and IBA, an important step in auxin homeostasis. The next step will be to determine whether or not these mutant lines demonstrate differential levels of IAA and IBA, a step that will allow for a more conclusive understanding of the role of these proteins in this biosynthetic step.

Quantitative Analysis

Calculating R-Values for Novel IBA and ICA Internal Standards

When biological samples are analyzed via GC-SIM-MS, the machine only detects the selected ions for each of the compounds measured, or m/z 130, 136, 189, and 195 for me-IAA, m/z 130, 139, 217 and 226 for me-IBA, and m/z 130, 139, 223 and 232 for me-ICA. Together, these measurements underestimate the actual abundance of the fragments these ions represent because they do not measure isotopes of the associated ion. Calculating the amount of the endogenous compounds relies upon sound ratios between

the compound and its respective internal standard. These two compounds will have a relatively constant abundance of isotopic ions. That is, whenever IAA is run on a GC-SIM-MS approximately 10% of its base peak (know as the 'quinolinium ion') is m/z 131 (where each C and N has a 1% chance of being an isotope), and approximately 1% of the base peak is m/z 132. GC-MS data analysis software does not account for this, it detects the selected M^+ peak at 100% abundance and measures all other peaks relative to it. However, this peak is actually representative of only ~89% of the quinolinuim ion present. This exact percentage must be determined for both the endogenous, unlabeled, compound and its respective internal standard because each endogenous compound will have a different isotopic envelope within the resulting ions when compared to its internal standard. We need to know what percentage of the base peak the M^+ abundance of both compound actually represented because we are comparing the relative abundance of M^+ of the internal standard to the endogenous compound in the same spectra where only one of these are taken for the individual base peak of that data set. Knowing the ratio between the percentage that the base peak of each compound allows for an appropriate correction of this selective ion monitoring. Importantly, R-values must be determined via GC-MS analysis for each endogenous compound/internal standard pair. Once determined, this value can then be used for every application of that internal standard.

The IAA internal standard used in these experiments, [$^{13}C_6$]-IAA, has a reported *R* value of 1.13 (Cohen et al., 1986). The R-values for the novel [$^{13}C_8$-^{15}N]-IBA and [$^{13}C_8$-^{15}N]-ICA internal standards was calculated with the data in Figure A4 in the Appendix with the following equation:

$$R_{value} = \frac{\left(\frac{m/z\ 130}{\sum m/z\ 130,\ 131,\ 132}\right)_{unlabeled}}{\left(\frac{m/z\ 139}{\sum m/z\ 130,\ 131,\ 132,\ 133,\ 134,\ 135,\ 136,\ 137,\ 138,\ 139}\right)_{labeled}}$$

The R-values for the [$^{13}C_8$-^{15}N]-IBA and [$^{13}C_8$-^{15}N]-ICA internal standards were calculated to be 1.33 and 1.32, respectively.

Percent yield of [$^{13}C_8$-^{15}N]-indole-3-butyric acid synthesis

The percent yield of [$^{13}C_8$-^{15}N]-IBA synthesized was determined by the following equation: $\frac{Mass_{\text{indolealkanoic acid (product)}}}{Mass_{\text{Indole (reactant)}}} \times 100$ where the mass of the [$^{13}C_8$-^{15}N]-indole used was 0.05g, and the mass of [$^{13}C_8$-^{15}N]-IBA product was calculated from the data in Figure A4 in the Appendix using the following equation:

$$Y = \left(\frac{C_i}{C_f} - 1\right) \times \frac{X}{R}$$

where Y is the amount of the unlabeled IBA (µg); X is the amount of labeled IBA (µg); C_i is the initial percentage of m/z 139 relative to 130 + 139, or 100%; C_f is the percentage of m/z 139 relative to the total ion current at m/z 130 and 139 found by the GC-SIM-MS, or 19.25%. Importantly, C_f accounts for both the naturally occurring ^{13}C isotopes and the partially enrichment of the isotopic indole through adding m/z 130, 131, and 132 for the

'm/z 130' numerator and adding m/z 136, 137, 138, 139, 140 for the 'm/z 139' denominator:

$$C_i = \frac{\text{m/z}(130+131+132)}{\text{m/z}(130+131+132) \; + \; \text{m/z}(136+137+138+139+140)}$$

X is the amount of labeled added, or 1 µg. *R* is the ratio of the fraction of endogenous IAA or IBA that has a peak at m/z 130 to the fraction of m/z 136 or m/z 139 for IAA and IBA, respectively. The amount of the IBA internal standard in the 5 µl added to the MS vial can be calculated:

$$\left(\frac{100}{19.25}-1\right)\frac{1}{1.33} = 3.2\mu\text{g in } 5\mu\text{l solution}$$

Thus, the percent yield and the total amount of IBA internal standard synthesized can be calculated:

$$\frac{3.2\ \mu\text{g (calculated amount in MS vial)}}{5\ \mu\text{l (total volume in MS vial)}} = \frac{7.68\ \text{mg (total mass of product}}{12\ \text{ml (total volume of product)}}$$

$$\text{Percent Yield} \;=\; \frac{\text{Mass}_{\text{indolealkanoic acid (product)}}}{\text{Mass}_{\text{indole (reactant)}}} \times 100 = \frac{7.68\ \text{mg}}{50\ \text{mg}} \times 100 = 15.36\%$$

Post-SPE Purification of Indolealkanoic acids

Methylation of [^{3}H]-IBA

The primary goal of the radiolabeled experiments was to track the elution of IBA through the post-SPE purification steps. This was needed because the guidelines previously described for this method of purification were for me-IAA only. [^{3}H]-IBA was added to samples after SPE and methylated. Samples were run on TLC with IBA and me-IBA samples. 5 mm sections were collected along the plate were collected and the radioactivity of each was assessed with a scintillation counter. Figure 14 displays the data from these radiolabeled experiments.

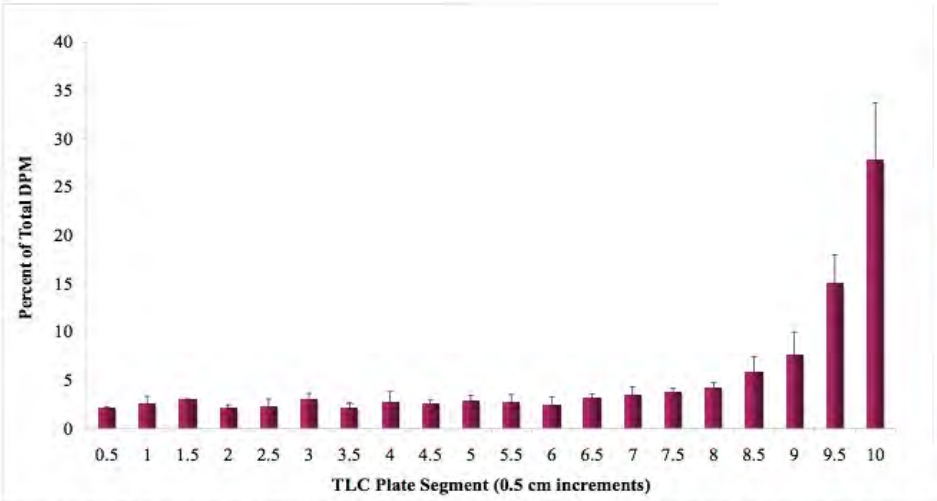


Figure a *[^{3}H]-IBA is successfully methylated with ethereal diazomethane.* TLC segments 9.-10.0 and 5-6 represent the regions of me-IBA and IBA migration, respectively. 0.5 cm segments of the TLC plate were collected and tested for radioactivity due to [^{3}H]. Data represents the average of four individual trials.

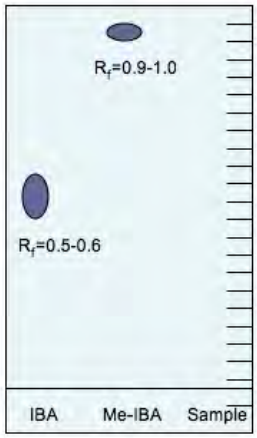


Figure b *Schematic of IBA and me-IBA on TLC.* Dashes represent regions collected for radioactivity measurements in Figure a. me-IBA and IBA were used to determine R_f regions.

Figure 14 *[^{3}H]-IBA is successfully methylated.* Samples of [^{3}H]-IBA were methylated with diazomethane after SPE-purification and run on a TLC plate. 0.5 cm segments of the TLC plate were collected and tested for radio activity from [^{3}H]. Figure a is the percent of total radioactivity (DPM) of each TLC segment collected in each trial. Figure b is a schematic of the TLC plate. Virtually all radioactivity was observed in the R_f region of me-IBA.

Post-SPE Purification Protocol

Once the robust methylation of [^{3}H]-IBA was established, the elution pattern of IBA under the IAA-specific post-SPE purification protocol was determined. Figure 15a shows that, as expected, virtually no me-[^{3}H]-IBA was present in the initial flow through and the 10%, 20%, and 30% washes. The 50% methanol elution step contained roughly slightly less than 40% of the total me-[^{3}H]-IBA and the majority of the me-[^{3}H]-IBA remained bound to the column.

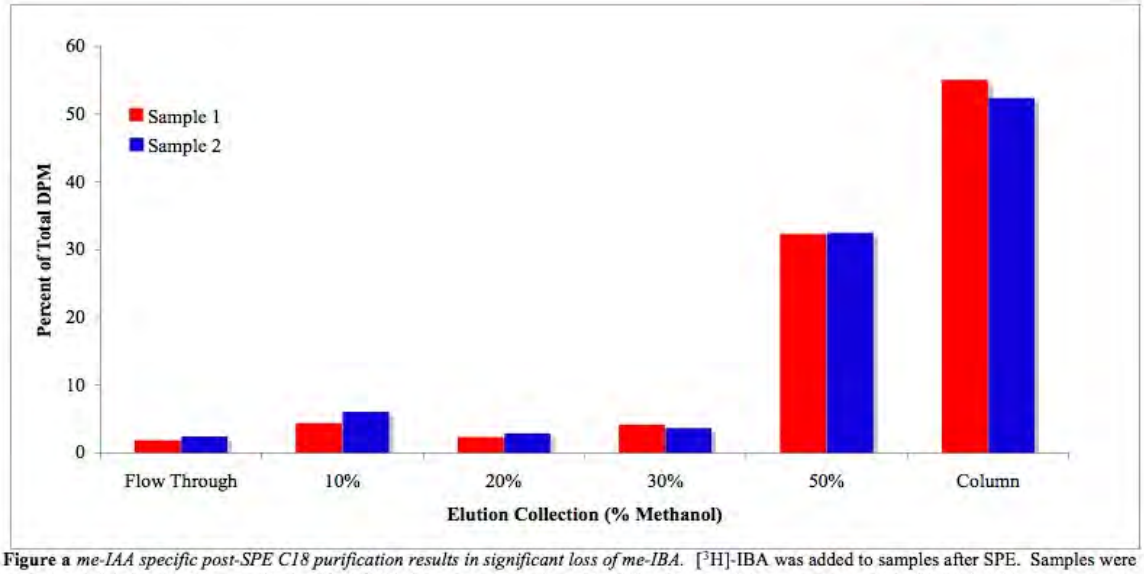


Figure a *me-IAA specific post-SPE C18 purification results in significant loss of me-IBA.* [^{3}H]-IBA was added to samples after SPE. Samples were methylated, dried under N_2, and dissolved in 0.2M imidazole and transferred to C18 columns. Columns were washed with 10%-30% methanol and eluted with 50% methanol. Radioactivity due to [^{3}H] were measured in each step. The majority of me-[^{3}H]-IBA remained in the column after the 50% methanol wash.

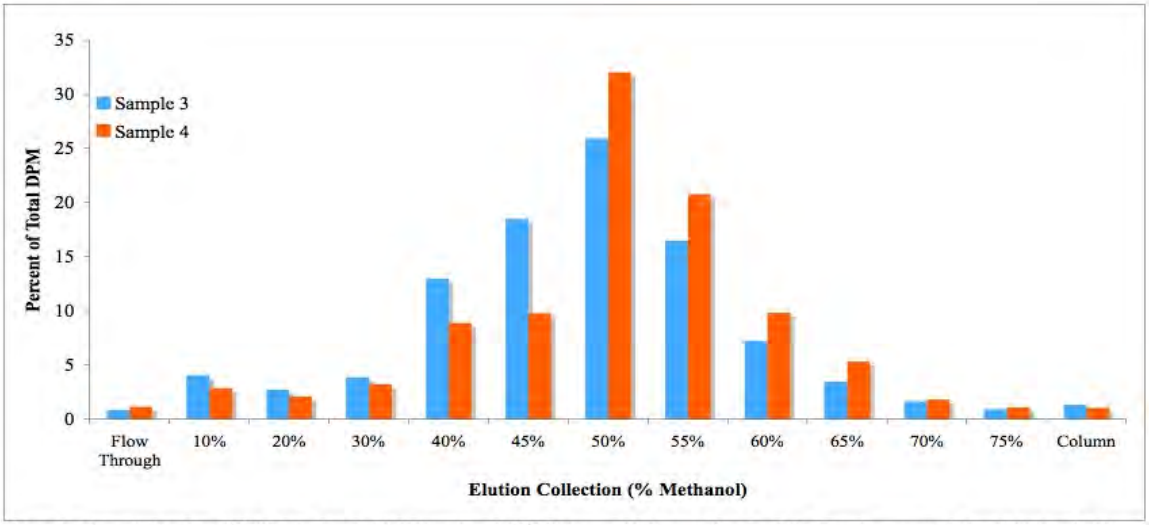


Figure b *The majority of me-[^{3}H]-IBA is eluted with 60% methanol.* [^{3}H]-IBA was added to samples after SPE. Samples were methylated, dried under N_2, and dissolved in 0.2M imidazole and transferred to C18 columns. Columns were eluted sequentially with increasing methanol dilutions. Radioactivity from me-[^{3}H] -IBA was measured in each step. 85% of the me-[^{3}H] -IBA was eluted with 60% methanol. Virtually no me-[^{3}H] -IBA remained in the column after 65% methanol.

Figure 15 *me-[^{3}H]-IBA is fully eluted from the C18 column with 60% methanol.* Figure a and b represent two separate experiments. Figure a shows that me-[^{3}H]-IBA is only partially eluted with 50% methanol. Figure b displays the elution pattern of me-[^{3}H]-IBA under a series of methanol dilutions.

The experiment resulting in Figure 15a was repeated eluting with a series of 10%, 20%, 30%, 40%, 45%, 50%, 55%, 60%, 65%, and 75% methanol solutions to precisely determine the elution pattern of me-[^{3}H]-IBA. Figure 15b shows that the majority (90%) of me-[^{3}H]-IBA was eluted by the addition of 60% methanol leaving only a small amount

of me-[^{3}H]-IBA still bound to the column. Thus, a simple solution to expand the IAA-specific post-SPE clean up protocol to include me-IBA is made clear. Given that both me-IAA and me-IBA are not eluted with the 30% wash (Barkawi et al., in preparation, and this work) samples can be washed with a 10%, 20%, and 30% methanol solution series and eluted with a 60% methanol solution. This protocol is especially important when quantifying both endogenous IAA and IBA within the same sample. By extension, this method could be applied to longer chain indolealkanoic acids, such as ICA.

Application of Expanded Post-SPE Purification Protocol

Post-SPE C18 solid phase purification of me-IAA has shown to greatly decrease the amount of contamination remaining within samples extracted from plant tissues after the initial automated solid-phase extraction. The C18 column has a relatively high affinity to the nonpolar me-IAA within the post-SPE solution. Figure 16 is an extracted chromatogram of one sample in which ions representative of me-IAA detection are monitored.

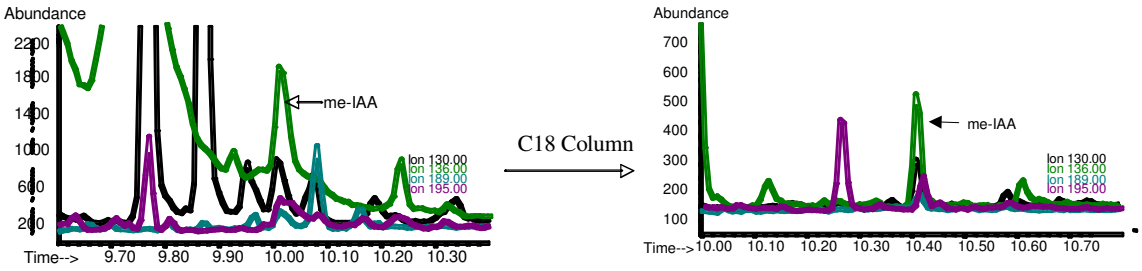


Figure a: *Abundance and retention of selected ions of SPE product.* Products of the SPE method where dried of N $_2$ and dissolved in ethyl acetate for GS-SIM-MS analysis. The above graph displays high backgrond values related to the presence of contaminants that were not fully removed via SPE.

Figure b *Abundance and retention times of further C18 purification post SPE.* Products remaining from GC-SIM-MS were resuspended in methanol and ran through a C18 column that selects me-IAA. Note the low background levels. This corresponds to the removal of contaminants. It should be noted that the retention time for me-IAA is shifted slightly. This is due to a recent replacement of the GC column.

Figure 16 *Post-SPE purification greatly reduces background contamination of samples with similar retention times as me-IAA.* Ion extracted chromatogram for me-IAA before and after (Figure a and b, respectively) C18 post SPE purification following the IAA-specific protocol. Each color line represents the retention times and associated abundance of each separate ion.

Precise calculations of free IAA and IBA are dependent upon a low background because they allow for the best relative measurements between each ion. This is less of an issue when trying to measure IAA because the amount of free IAA is generally twice that of IBA and will have ion peaks that rise above the background contamination enough to make significant calculations of free IAA (see Barkawi et al., in preparation; Ludwig-Müller et al., 1993). Getting precise IBA measurements presents a more difficult problem mainly because it exists in very low concentrations that are often indistinguishable from background readings under normal extraction protocols (see Figure 17a). The basic concept behind the C18 clean up is to take advantage of the slightly hydrophobic nature of me-IAA and me-IBA. Washing with a series of methanol dilutions will clean the column of contaminants. A high % of methanol will compete with the methylated indolealkanoic acid and eventually lead to the elution of the product. Because me-IBA is slightly more hydrophobic with its longer side chain it was necessary to adjust the elution pattern to maximize the elution of me-IBA without considerable lost of me-IAA. Thus, a higher percentage of methanol solution was needed to fully elute IBA from the column. Figure 17 displays the efficiency of eluting the column with a great percentage of methanol to extract IBA from the column.

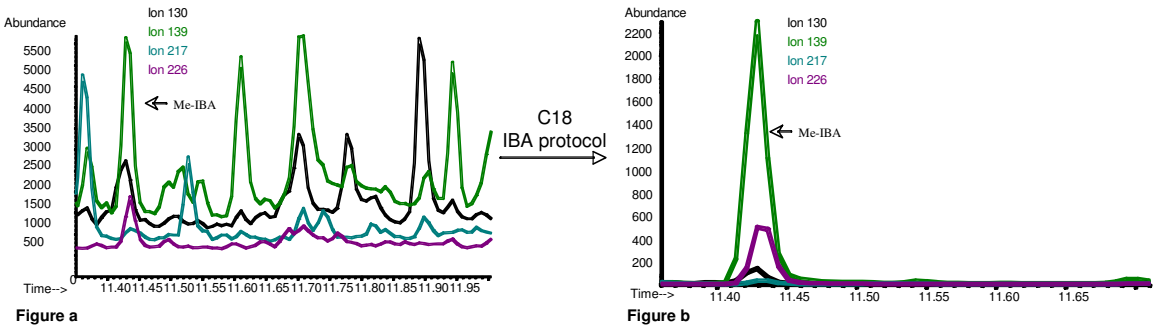


Figure 17 *IBA-expanded protocol greatly reduced amount of sample contamination.* Ion extracted chromatogram for me-IBA before (Figure a) and after C18 post-SPE clean up following the IBA-expanded protocol (Figure b). Each color line represents the retention times and associated abundance of each separate ion. The C18 column will attract the non-polar me-IAA and me-IBA. After samples are added to the C18 columns, they are washed with low concentrations of methanol and eluted with high concentration of methanol.

Importantly, the IBA-expanded protocol mentioned above does not reduce the amount of me-IAA that is eluted from the column because the only major alteration of the protocol is increasing the percentage of methanol the column is eluted with after the washes. A higher percentage of methanol will elute both IAA and IBA. This simple addition to the automated SPE method described above ensures samples with very low contamination at the cost of very minimal product loss.

Quantification of IAA and IBA in *Arabidopsis thaliana*

Free IAA and IBA in Arabidopsis *under different growth conditions*

Ludwig-Müller (2007) demonstrated that IBA synthetase activity varies under different experimental growth conditions, within different ecotypes, and as the seedlings ages. Given that IBA synthetase is essential in the production of IBA from IAA, it follows that activity assays for this protein should, at least in part, serve as predictors of changing IBA levels, and, by extension, IBA activity. However, this method doesn't rule out the production of IBA via different pathways that are active under different conditions. Indeed, IAA synthesis can follow numerous pathways (Cohen et al., 2003)

and can be produced through the hydrolysis of IAA-amino conjugates (Bialek and Cohen, 1986). The indirect method to detect IBA employed by Ludwig-Müller (2006) would not account for the accumulation of IBA from other pathways. IBA can exist in conjugated form in wheat (Campanella et al., 2004). The ideal assay for IBA activity would be the direct measurement of free and conjugated IBA within samples.

The basic goal of expanding the IAA quantification protocol to be able to accurately measure IBA within the sample is to determine the relative amounts of these compounds in mutants to screen for their potential role in synthesizing IBA from IAA. Prior to pursuing the quantification of these auxins in mutants, baseline data from various ecotypes of wild type *Arabidopsis* seedlings grown under different experimental conditions is needed to be established in order to assess the optimal conditions in which IBA is present and to determine variability.

Col-0 seedlings were sown in both liquid culture and on solid growth media and harvested at 3, 5, and 9 days after planting. IAA and IBA measurements were calculated and are presented in Figure 18 and Figure A5 in the Appendix.

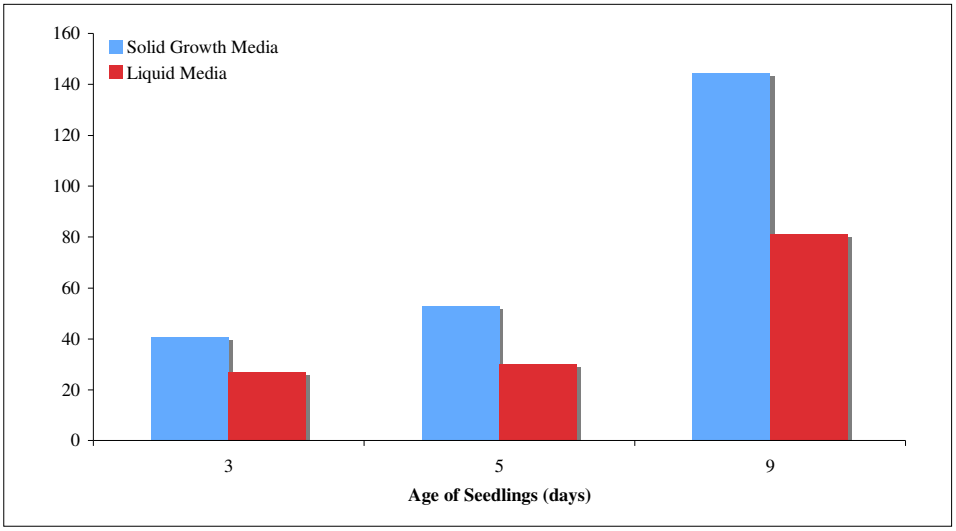


Figure a

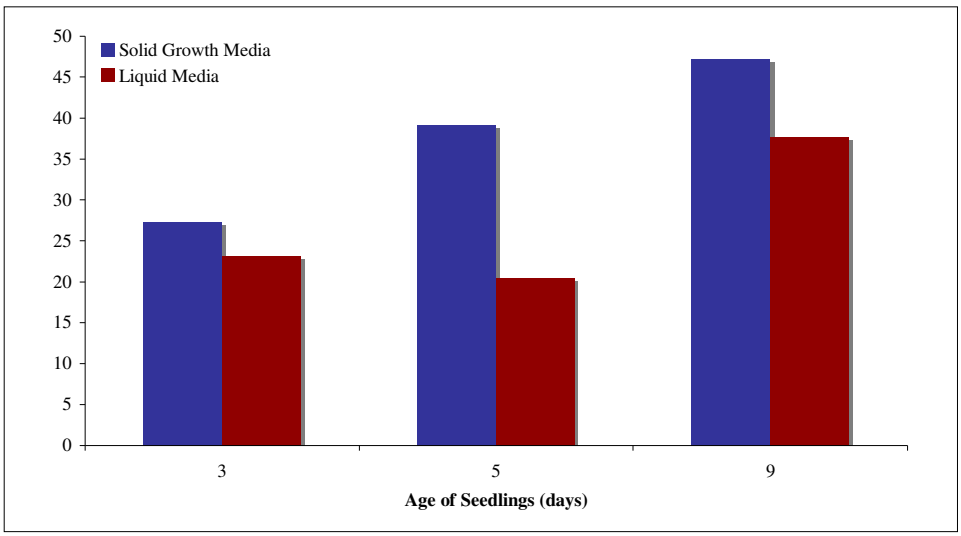


Figure b

Figure 18 *Arabidopsis seedlings have higher concentrations of free IAA and IBA when grown on solid growth media.* Figures a and b represent free IAA and IBA in Col-0 *Arabidopsis* seedlings grown on solid and liquid grown media, respectively. Note: an increasing amount of IAA and IBA internal standard were added to samples in Figures a and b leading to an apparent increase in free IAA and IBA over time.

It should be noted that the amount of internal standard within this trial increased proportionally to the mass of sample to have concentrations of 20 ng and 10 ng of IAA and IBA, respectively, per gram fresh weight of tissue. A follow up experiment was performed to determine the variation between IAA and IBA levels within the Col-0, Col-

4 and Ler ecotypes grown in liquid culture and on solid growth media over time. Figure 18 represent the amount of free IBA ad IAA within these samples, respectively.

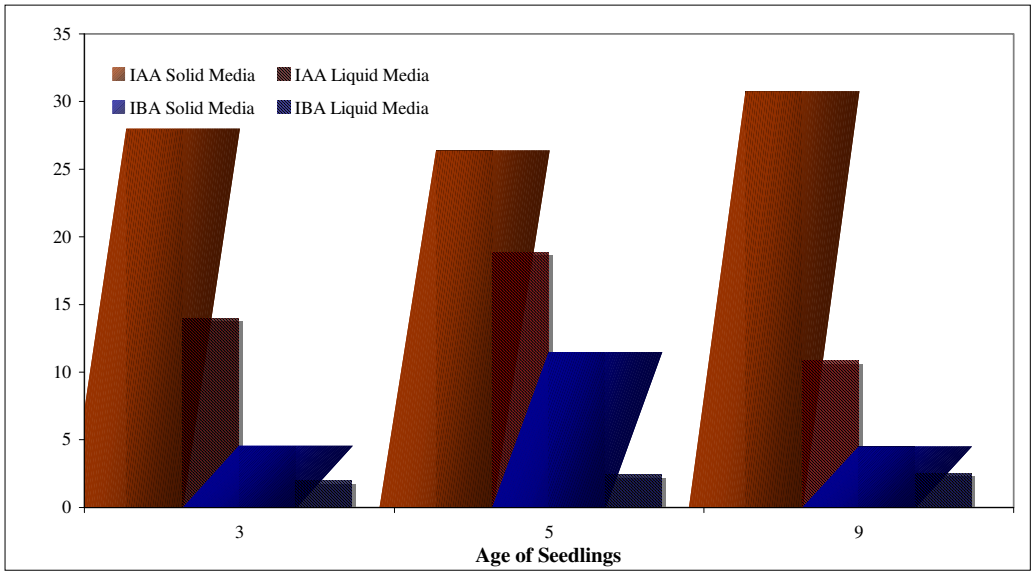


Figure 19 *Arabidopsis seedlings grown on solid media for 5 days is a suitable growth conditions for IAA and IBA quantification*. Col-0 seedlings were grown in liquid media or on solid media for 3, 5, and 9 days under continuous light at 24˚C. Liquid cultures were shaken at 150 rpm.

There are some important learning points from comparing each of these sets of data. The apparent variation between these two trials seems to be due to the amount of internal standard (See Figure A5 in the Appendix). The experiment presented in Figure 18 had more internal standard added by a factor of 2. It has been hypothesized that including such a relatively large amount of internal standard will upset the extraction and purification behavior of IAA and IBA via mass action effects (Lana Barkawi, personal communication). However, these data have relative, if no absolute, value. In both experiments, there is a marked increase of both IAA and IBA production within the seedlings grown on solid growth media. Furthermore, Figure 19 demonstrates that IBA production seems to peak slightly at 5 days and taper off slowly after that.

The apparent increase in detected IAA in Figure 18 is most likely due to the addition of a high amount of internal standard rather than having biological origins. However, it should be noted that these values are not outside of the reported biological variation within *Arabidopsis*. Together with the experiment measuring IAA and IBA within different ecotypes, the importance of adding equal amounts of internal standards within sets of samples is apparent. Together, these data help stream line a protocol in which to grow and assess the relative amounts of IAA and IBA within the mutant insertions lines. Thus, growing seedlings for 5 days on solid growth media will yield high enough IBA and IAA concentrations to quantify using the methods described in this paper.

Free IAA and IBA in Arabidopsis *mutants with altered adventitious rooting*

The primary aim of this research was to apply this modified method of IBA and IAA quantification gain insight into the biosynthetic relationship between these two auxins. Once optimal growth conditions, extractions and quantification parameters were tailor to accommodate the measurement of both IAA and IBA within the same sample, this technology was successfully employed to quantify IAA and IBA levels within mutants previously described. Seedlings from each insertion line and Col-0 were grown under continuous light on solid growth media and harvested five days after germination. Each line was grown in triplicate. In order to ensure consistent germination initiation, and therefore consistent quantification data, the plates were placed in 4˚C for 25 hours prior to being transferred to the normal growth conditions described above. Figure 20 and Table 3 shows the free IAA and IBA within these lines.

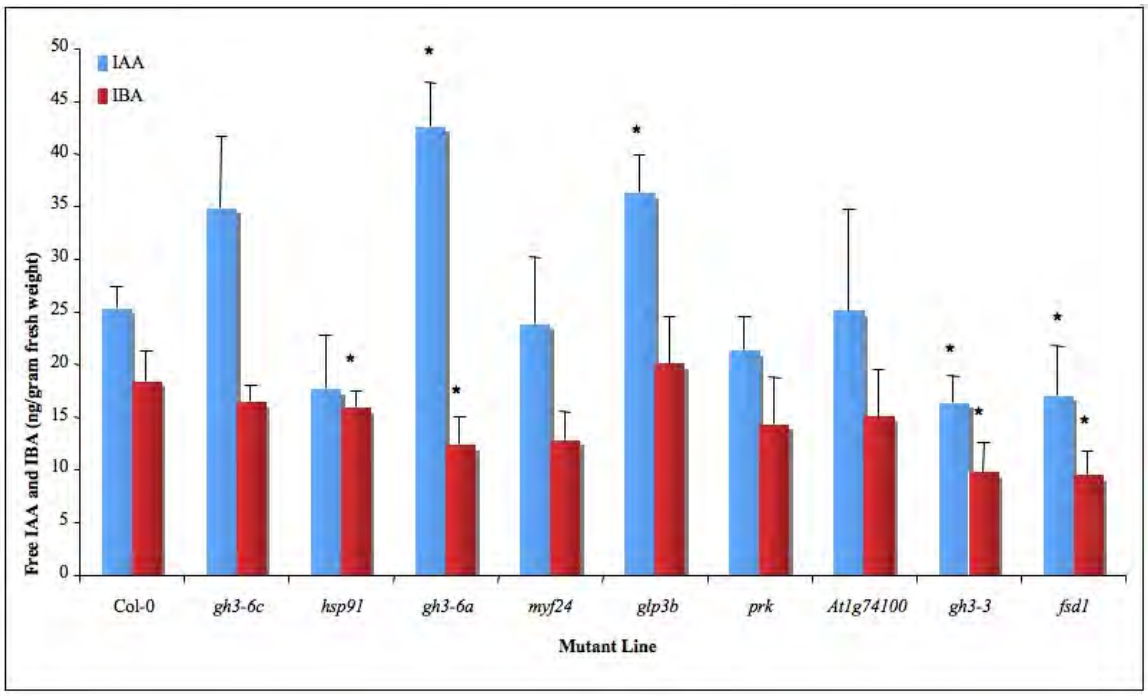


Figure 20 *Free IAA and IBA within insertion lines.* Seedlings were grown on solid media for fives under continuous light at 24˚C and harvested on day 5. Each data point represents the average of three independent trials, error bars are the standard deviate of the mean. Asterisks denote data points that are statistically significant when compared to Col-0 (p=0.05).

	Free IAA (ng / g fresh weight)	Standard Deviation	p-value	Free IBA (ng / g fresh weight)	Standard Deviation	p-value	Ratio of IAA:IBA
Col-0	25.32	1.96	-	18.36	2.80	-	1.38
gh3-6c	34.79	6.75	0.091	16.34	1.55	0.170	2.13
hsp91	17.61	5.08	0.086	15.83	1.60	0.042	1.11
*gh3-6a***	42.58	4.11	0.003	12.33	2.60	0.013	3.45
myf24	23.74	6.39	0.380	12.80	2.60	0.036	1.85
glp3b	36.25	3.57	0.003	19.99	4.50	0.240	1.81
prk	21.23	3.18	0.018	14.30	4.40	0.123	1.48
At1g74100	25.06	9.57	0.479	14.99	4.40	0.289	1.67
*gh3-3***	16.33	2.46	0.035	9.70	2.74	0.036	1.68
*fsd1***	17.05	4.61	0.018	9.45	2.23	0.004	1.80

Table 3 *Free IAA and IBA within insertion lines.* Free IAA and IBA measurements represent the average of three independent trials. T-test comparing the measurements from each insertion line to the Col-0 wild type was used to generate the p-values. Double asterisks denote insertion lines that demonstrated significantly different IAA and IBA levels compared to Col-0.

IAA levels within the Col-0 wild type were consistent with normal physiological ranges (see Figure18c) and IBA levels were nearly twice as high as that reported in Figure 19. This discrepancy is most likely due the stage of development at which these seedlings

were harvested. Each line in this experiment initiated germination within 24 hours of each other and demonstrated a very high germination rate (>90%). Germination rates were calculated by dividing the number of emerging seedlings by the number of total seeds sown. Col-0 seedlings harvested for the data presented in Figure 19 demonstrated a relatively low germination rate (~60%) and appeared to be somewhat underdeveloped compared to the insertion lines used in experiment above. Although this subjective assessment does not completely reconcile this different, the robust IBA levels measured in the insertion lines grown for 5 days (Figure 20; Table 3) demonstrated that a 5 day harvest time is suitable for IBA and IAA concentration measurements.

Notable deviations from these baseline wild type measurements displayed in Figure 20 are *gh3-6c, gh3-6a, gh3-3,* and *fsd1*. *gh3-6a*, *gh3-3*, and *fsd1* showed a significant difference in both IAA and IBA levels. Furthermore, *gh3-6a* and *gh3-6c* demonstrated a 2- and 3-fold increase in the ration between the amounts of IAA to IBA. This latter metric is perhaps the most revealing with respect to the role of these two genes within the conversion of IBA from IAA because mutant lacking a protein that is involved with the conversion of IAA to IBA would be expected to display a high ratio of IAA to IBA. Figure 21 displays the ratio of IAA to IBA within the insertion lines.

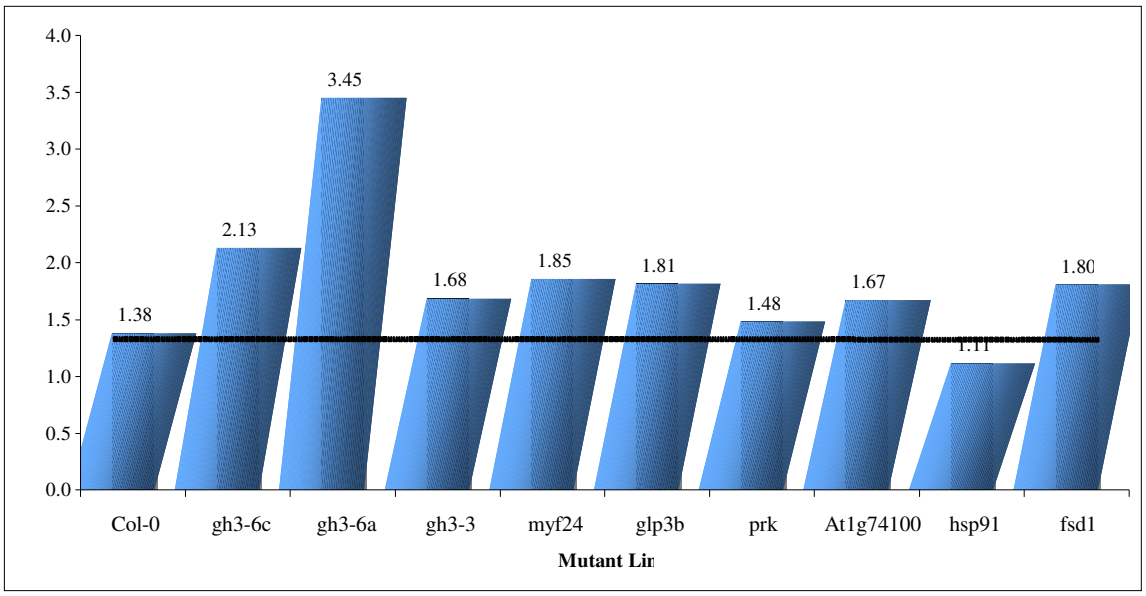


Figure 21 gh3-6 *mutants demonstrated an increase ratio of IAA:IBA*. Average levels of IAA were compared to the average levels of IBA within each insertion sample. The dashed line represents the ratio of [IAA]:[IBA] within Col-0 seedlings.

The *gh3-6* is known to conjugate IAA to amino acids, a mechanism utilized by *Arabidopsis* to maintain suitable IAA concentrations (Staswick et al. 2005). The data presented in Figures 20 and 21 supports these findings in that lines lacking this gene will be unable to conjugate excess IAA to amino acids leading to an increase in the free IAA pool.

The data presented in Figures 20 and 21 expand on the finding of Sorin et al. (2006) in that this work serves to further differentiate on the behavior of GH3 proteins within IAA homeostasis. In their assessment of GH3 protein expression within *Arabidopsis* lines that over or under express IAA (*sur2* and *ago1*, respectively) Sorin et al. (2006) observed similar expression patterns and intensities of GH3-3, GH3-5, and GH3-6 (Figure 3 in Sorin et al. 2006). The data presented in Figures 20 and 21 of this work suggest that although these proteins respond similarly to IAA, they act to maintain IAA homeostasis differently. Where the *gh3-6* mutants demonstrate an increased ratio of

IAA to IBA primarily due to the 2- to 3-fold increase of IAA within these lines, the *gh3-3* mutant had considerably less IAA as well IBA while maintaining a ratio of IAA to IBA that does not deviate significantly from that of Col-0. These results suggest that the *gh3-3* mutation effects both IAA and IBA synthesis and a divergent role of *GH3-6* and *GH3-3* in maintaining auxin homeostasis.

CONCLUSION

This thesis presents data surrounding the development and application of a high throughput method for IAA and IBA quantification within *Arabidopsis thaliana* seedlings. This work is important in laying the groundwork for assessing the complex relationship between IAA and IBA within plants as it presents a thorough protocol in quantifying their respective concentrations in the same plant tissue. Furthermore, this work assayed insertion lines mutated in genes correlated with auxin homeostasis and/or adventitious rooting that serve as potentially candidates for playing a role in the most direct redirect relationship of IAA and IBA, the production of IBA from IAA. Important studies have yet to be done in order to establish this relationship under various conditions.

This work reports the synthesis and application of a novel [$^{13}C_8$-^{15}N]-IBA internal standard and the synthesis of [$^{13}C_8$-^{15}N]-ICA. The latter of which can be now used to detect the presence of ICA within various plant species. Through observing the behavior of IBA throughout the methylation and post-SPE steps of indolealkanoic acid purification through a series of radiolabeled experiments this work was able to make adjustments to existing extraction protocols to allow for the easy purification and analysis of IBA and IAA within *Arabidopsis* seedlings. This method can be applied to a wide range of plant species and will be helpful in understanding the biological importance of IBA. The availability of [$^{13}C_8$-^{15}N]-IBA internal standard will be instrumental in research involved IBA quantification. Given the concentrations needed to within GC-MS quantification protocols, the amount of this internal standard synthesized in this work will be more than enough for thousands of such measurements. I will be sending out samples of this

internal standard to researchers that are focused on IBA quantification, most notably to Prof. Dr. Jutta Ludwig Müller and Dr. Jennifer Normanly who are both actively working with their colleagues to either identify the enzymes involved in IBA synthesis and/or measuring IBA levels in plants.

There are many important steps that will need to be taken to properly identify and characterize the enzyme that catalyzes the first step in the conversion of IBA from IAA, such as measuring the relative concentrations of IBA and IAA within mutant lines described in this research. This research is ongoing and doing such quantification analysis is my next primary objective.

In order to verify the effectiveness of using the IAA/IBA quantification method employed in this research an IBA synthetase mutant will need to generated and tested in order to have a sound positive control for these mutant screens. No such mutant currently exists.

Currently, the role of GH3 proteins stands as an intriguing possibility in their role in catalyzing this reaction. Currently, less than half of the GH3 family has been well characterized in terms of their biological activity (Staswick et al., 2003; Park et al., 2007). What is known is they all are up-regulated in response to auxin (Staswick et al., 2003) and some conjugate amino acids to IAA. Figure 19 and Table 3 present quantification data of select insertion lines. These data highlight GH3-6 as a potential candidate as an enzyme that converts IAA into IBA. This protein is known to conjugate amino acids to IAA (Staskwick et al., 2003). Together, two hypotheses of this observed increase in the ratio between IAA and IBA can be drawn in respects to the role of the GH3-6 protein in IBA biosynthesis. This protein could be directly involved in both

conjugating amino acids to IAA as well as the conversion of IAA to product X. Alternatively, the decrease in the amount of IAA conjugates that would be expected in a *gh3-6* mutant could disrupt the conversion of IBA from IAA. The latter hypothesis suggests there to be a feedback mechanism that monitors IAA homeostasis through the production of its conjugates where an increase of conjugates would signal further sequestering of IAA into its more stable IBA derivative. It is difficult to determine exactly what role GH3-6 has in the conversion of IBA to IAA with this data. However the data presented in this research certainly merits further investigation into the specific role that this protein has in this important biosynthetic pathway of IBA.

Given the high redundancy within the *Arabidopsis* genome it will be important to create multiple *GH3* gene mutants in which closely related *GH3* genes will be loss of function. I am currently working on cultivating a double knockout mutant that is homozygous for the SALK_151766 and SALK_08253 insertion with hopes of generating a *gh3-5/gh3-6* mutant. Measuring the IAA and IBA concentration within this mutant will address the potential redundancy within this family of proteins in the IAA to IBA pathway.

Confirmation of the enzyme that catalyzes the production of product X is dependent upon two steps. First, product X will need to be isolated and fully characterized. Second, candidate proteins will need to be purified and incubated with necessary reactants to produce product X. Both of these steps are achievable with little resource expenditure.

There is also important work yet to be done in terms of applying the IBA/IAA quantification method developed within this research. This research presented

phenotypic data on a series of mutants lacking genes indicated in auxin homeostasis. In order to better understand the role of IBA within adventitious rooting, hypocotyls and root elongation, it will be important to contextualize these phenotypic characteristics within IBA over-and under-producers. Once this relationship is established, it will be possible to develop more accurate IBA-responsive assays that do not rely on the application of synthetically produced IBA and will thus have greater biological pertinence. Furthermore, this work demonstrated the ability to accurately measure IBA and IAA within the same sample to address a key element within the biosynthetic pathway of IBA. The application of this technology to insertion lines provided evidence as the potential importance of GH3 proteins within this biosynthetic pathway.

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APPENDIX

T-DNA insertion	Location of Insertion	Mutant Name	Genomic 3' (LP) Primer sequence
CS104933	Exon	*gh3-3*	TTTCAAGAATTAGGGTTTAAGGTTTCTGA
SALK_033434	Exon	*gh3-5a*	TTGGATTCGGACTTGATGAAC
SALK_151766	Exon	*gh3-5a*	CGTTCAAGATGACCAAATCTG
SALK_082530	Exon	*gh3-6a*	AAAAATAAAACTATTAGAATGCAGCAAG
SALK_140227	1000-Promoter	*gh3-6b*	TGTGTTCACACGAAACAAAATG
SALK_023621	Exon	*gh3-6c*	CTCAGGCCAATGTTTCTCAAG
SALK_060813	Exon	*gh3-6d*	CGCTTAGAGAAACATAAACCGG
SALK_045354.47.75.x	Exon	*myf24*	TTGGATAATCGCTTGTAGACTTG
SALK_000898.50.00.x	Exon	-	TCCATCCAACAAGATCTCTGG
SALK_082815.45.00.x	Exon	*hsp91*	TCTTTACACGCTCCAAAATCG
SALK_003961.56.00.x	Exon	-	ACCAAGCCTCTCTCTACCTGC
SALK_029455.51.70.n	Exon	*fsd1*	CGCATGTATACATTTTCATGTGTC
SALK_055557.55.50.x	Exon	*glp3b*	TCAACCTCGAGAGTGATGGTC
SALK_076352.55.75.x	Exon	*prk*	ATTTTACCGTTTGATGCGTTG
WiscDsLox368E04	Exon	*bip1*	GAGGGGTTCCTCTGTCAAAAC

Figure A1 Left and right primers used for genotyping of insertion lines. All insertion lines used in this study were T-DNA knock-out mutations.

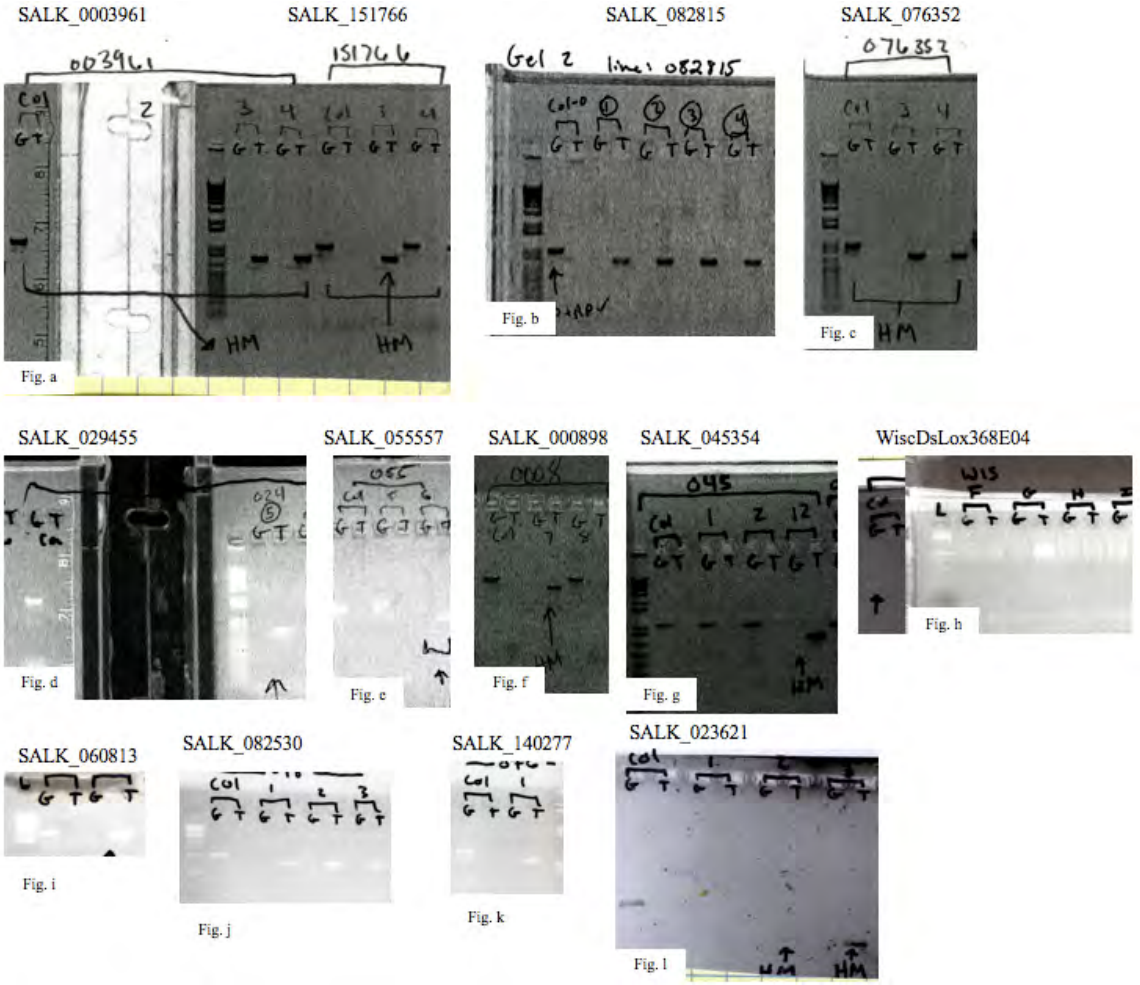

Figure A2 *PCR genotype analysis of heterozygous F_1 progeny reveal homozygous lines.* Lines homozygous (HM) for the T-DNA insertion were identified through the amplification of regions flanked by

primers specific for genomic DNA (“G”) and the T-DNA insert (“T”). Both “G” and “T” primer pairs shared the same genomic RP primer. Wild type Col-0 was used to check “G” primer pairs specific to each insertion line and gene pair. Figure a displays two plants HM for the SALK_0003961 insert and one plant HM for the SALK_151766 insert. Figure b displays four lines HM for the SALK_082815 insert, Figure c displays two lines HM for the SALK_076352 insertion. Figure d show two gels: the left gel displays the Col-0 primer pair for the SALK_029455 insert showing product for genomic region amplification only and the gel on the right shows a plant HM for the same insert. Figure e displays a plant HM for the SALK_055557 insert in lanes 5 and 6. Figure f shows a plant HM for the SALK_000898 insert in lanes 3 and 4. Figure g shows a plant HM for the SALK_045345 insert in lanes 7 and 8. Figure h shows two gels: the gel on the left the Col-0 primer pair for the WisDsLox368E04 insert and the gel on the right shows a plant HM for this insert in lanes 4 and 5. Figure i shows a plant HM for the SALK_060813 insert in lanes 4 and 5. Figure j shows three plats HM for the SALK_082530 insert. Figure k shows a plant HM for the SALK_140277 and Figure l shows two lines HM for the SALK_023621 insert. Once HM plants were identified, they were grown to maturity after which they were dried out and their seeds were harvested for the seed stocks used in this study.

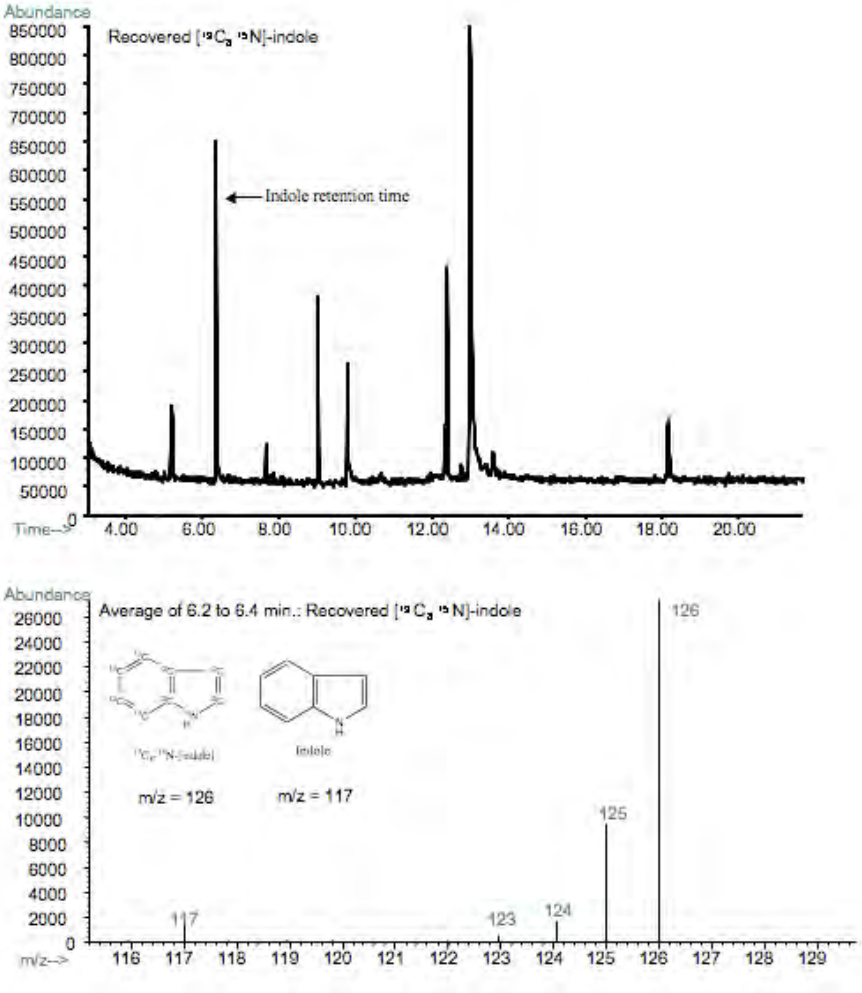

Figure A3 *GC-SIM-MS spectra of recovered indole from IBA internal standard synthesis*. The m/z represent the molecular ion of the fully enriched indole and m/z 117 the molecular ion of unlabeled indole.

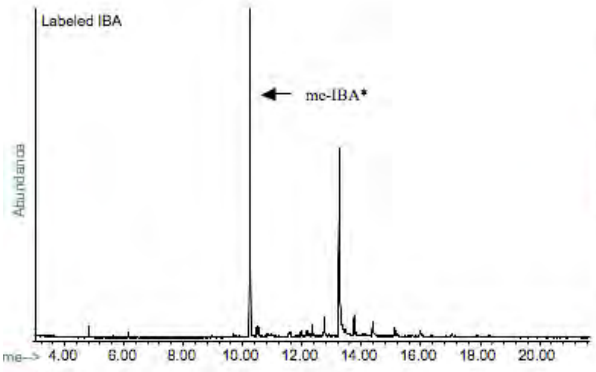

Figure a

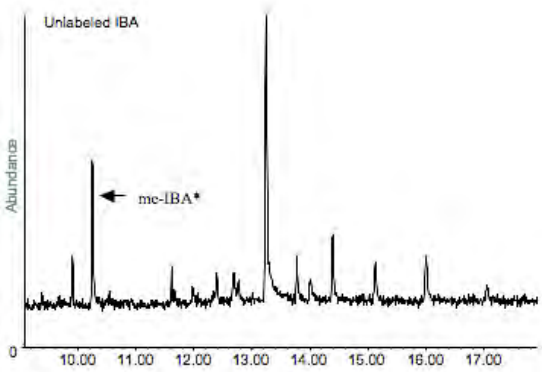

Figure c

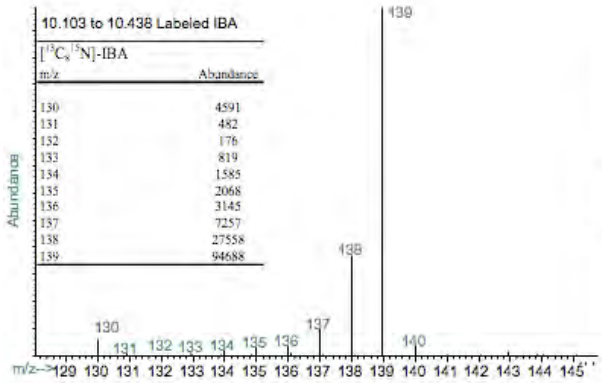

Figure b

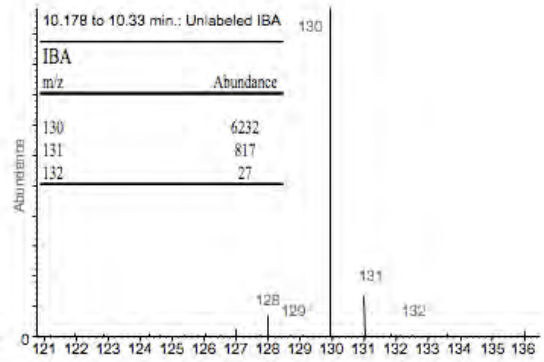

Figure d

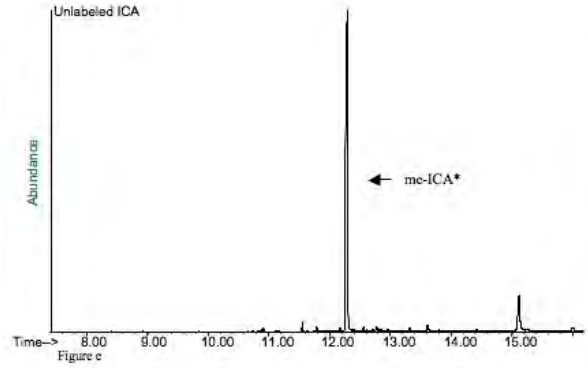

Figure e

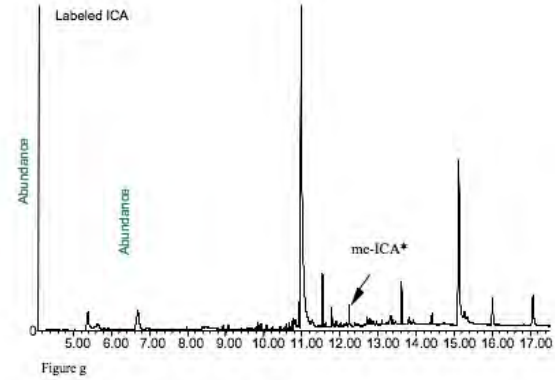

Figure g

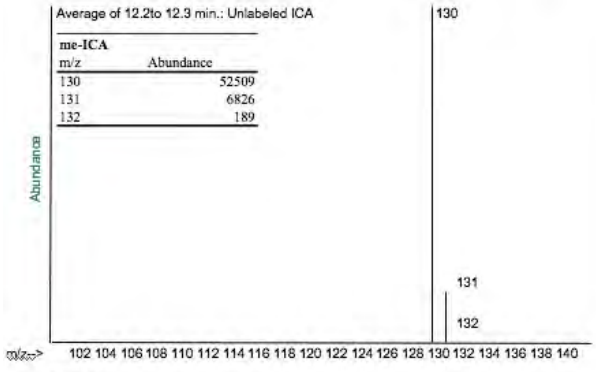

Figure f

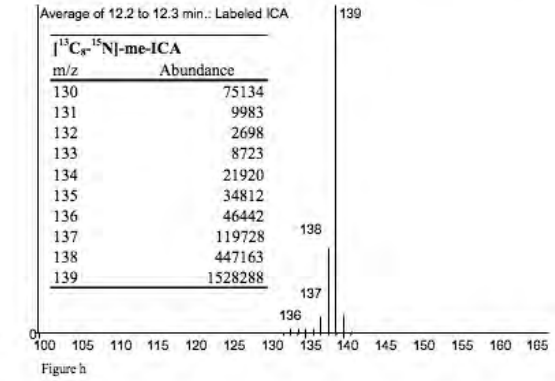

Figure h

Figure A4 *GC-MS spectra for [$^{13}C_8{}^{15}N$]-IBA and IBA used to calculate the R-value for the [$^{13}C_8{}^{15}N$]-IBA internal standard.* Both compounds were methylated prior to GC-MS analysis. Figures a and c are the GC-MS chromatogram of the samples. me-IBA have a retention time of 10.1 to 10.4 minutes for both labeled and unlabeled products. Figures b and d are the MS bar graphs showing the ion abundance within each sample. The inset tables in Figures b and d shows the tabulated abundance of the selected ions used to calculate C_i value and the R-value for the equation used in calculating the amount of labeled IBA synthesized. Figures e-f are the GC-MS spectral data and tabulated results used in the calculation of C_i value and the R-value for the ICA internal standard.

Col-0 seedlings grown in solid or liquid growth media (IAA)					
Sample Name	m/z 130	m/z 136	fresh weight	Amount $^{13}C_6$-IAA internal standard added (ng)	Free IAA (ng / g fresh weight)
3 day GM	2164	91429	0.0238	46.368	40.81
3 day liquid	1940	91810	0.032	46.368	27.10
5 day GM	1669	58795	0.076	160.22	52.96
5 day liquid	2664	106560	0.1178	160.22	30.09
9 day GM	1651	22524	0.1185	264.13	144.58
9 day liquid	2329	33459	0.2006	264.13	81.11

Col-0 seedlings grown in solid or liquid growth media (IBA)					
Sample Name	m/z 130	m/z 139	fresh weight	Amount IBA internal standard added (ng)	Free IBA (ng / g fresh weight)
3 day GM	652	8826	0.0238	9.968	23.26
3 day liquid	584	6951	0.032	9.968	19.68
5 day GM	453	4652	0.076	34.532	33.27
5 day liquid	750	9502	0.1178	34.532	17.40
9 day GM	532	4798	0.1185	56.96	40.07
9 day liquid	575	3829	0.2006	56.96	32.06

Figure a

Different ecotypes grown in solid or liquid growth media (IAA)					
Sample Name	m/z 130	m/z 136	fresh weight	Amount $^{13}C_6$-IAA internal standard added (ng)	Free IAA (ng / g fresh weight)
3d solid media Col-0	-	-	-	-	-
3d solid media Col-4	140	69	0.0289	1	62.13
3d liquid media Col-4	319	199	0.097	1	14.62
3d solid media Ler	512	235	0.04	1	48.20
5d solid Col-0	130	79	0.0552	1	26.38
5d liquid media Col-0	1102	595	0.0868	1	18.88
5d solid media Col-4	648	250	0.1089	1	21.06
5d liquid media Col-4a	1135	403	0.1034	1	24.10
5d liquid media Col-4b	1330	547	0.1186	1	18.14
5d solid media Ler	2061	1111	0.0247	1	66.46
9d solid media Col-0a	1220	724	0.049	1	30.43
9d solid media Col-0b	709	281	0.0717	1	31.14
9d liquid media Col-0a	1920	3554	0.1857	1	2.57
9d liquid media Col-0b	1759	714	0.2276	1	9.58
9d solid media Col-4a	1899	834	0.0338	1	59.62
9d solid media Col-4b	124	47	0.0354	1	65.95
9d liquid media Col-4a	-	-	-	-	-
9d liquid media Col-4b	613	333	0.1278	1	12.75
9d solid media Lera	1040	431	0.0627	1	34.06
9d solid media Lerb	2277	1085	0.0661	1	28.10

Different ecotypes grown in solid or liquid growth media (IBA)					
Sample Name	m/z 130	m/z 139	fresh weight	Amount IBA internal standard added (ng)	Free IBA (ng / g fresh weight)
3d solid media Col-0	-	-	-	-	-
3d solid media Col-4	-	-	-	-	-
3d liquid media Col-4	20	20	0.097	0.5	4.56
3d solid media Ler	16	45	0.04	0.5	3.93
5d solid Col-0	19	26	0.0552	0.5	5.86
5d liquid media Col-0	50	105	0.0868	0.5	2.43
5d solid media Col-4	130	46	0.1089	0.5	11.48
5d liquid media Col-4a	70	68	0.1034	0.5	4.41
5d liquid media Col-4b	73	131	0.1186	0.5	2.08
5d solid media Ler	129	196	0.0247	0.5	11.79
9d solid media Col-0a	117	328	0.049	0.5	3.22
9d solid media Col-0b	45	67	0.0717	0.5	4.14
9d liquid media Col-0a	1040	159	0.1857	0.5	15.59
9d liquid media Col-0b	877	158	0.2276	0.5	10.79
9d solid media Col-4a	88	226	0.0338	0.5	5.10
9d solid media Col-4b	6	19	0.0354	0.5	3.95
9d liquid media Col-4a	-	-	-	-	-
9d liquid media Col-4b	19	48	0.1278	0.5	1.37
9d solid media Lera	33	124	0.0627	0.5	1.88
9d solid media Lerb	109	228	0.0661	0.5	3.20

Figure b

Figure A5 Raw data used in IAA/IBA quantification of different ecotypes of *Arabidopsis* grown under different growth conditions over time for Figures 18 in the text. Dash marks indication loss of sample or inconclusive data points due to low tissue mass.

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Transport of the Two Natural Auxins, Indole-3-Butyric Acid and Indole-3-Acetic Acid, in Arabidopsis[1]

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Polar transport of the natural auxin indole-3-acetic acid (IAA) is important in a number of plant developmental processes. However, few studies have investigated the polar transport of other endogenous auxins, such as indole-3-butyric acid (IBA), in Arabidopsis. This study details the similarities and differences between IBA and IAA transport in several tissues of Arabidopsis. In the inflorescence axis, no significant IBA movement was detected, whereas IAA is transported in a basipetal direction from the meristem tip. In young seedlings, both IBA and IAA were transported only in a basipetal direction in the hypocotyl. In roots, both auxins moved in two distinct polarities and in specific tissues. The kinetics of IBA and IAA transport appear similar, with transport rates of 8 to 10 mm per hour. In addition, IBA transport, like IAA transport, is saturable at high concentrations of auxin, suggesting that IBA transport is protein mediated. Interestingly, IAA efflux inhibitors and mutations in genes encoding putative IAA transport proteins reduce IAA transport but do not alter IBA movement, suggesting that different auxin transport protein complexes are likely to mediate IBA and IAA transport. Finally, the physiological effects of IBA and IAA on hypocotyl elongation under several light conditions were examined and analyzed in the context of the differences in IBA and IAA transport. Together, these results present a detailed picture of IBA transport and provide the basis for a better understanding of the transport of these two endogenous auxins.

Auxins are phytohormones involved in mediating a number of essential plant growth and developmental processes. The majority of the research conducted on endogenous auxin has focused on the primary free auxin in most plants, indole-3-acetic acid (IAA), yet there are other abundant auxins in plants. Indole-3-butyric acid (IBA) comprises approximately 25% to 30% of the total free auxin pool in Arabidopsis seedlings (Ludwig-Muller et al., 1993). Although there have been great advances in understanding the molecular mechanisms behind IAA action and transport (Muday and DeLong, 2001; Friml and Palme, 2002; Leyser, 2002), it is not yet clear whether IBA and IAA act and move by similar mechanisms.

In vivo studies on the function of IBA are rather limited (Ludwig-Muller, 2000; Bartel et al., 2001). IBA has been identified in a number of plant species from maize (*Zea mays*) and pea (*Pisum sativum*) to Arabidopsis, and concentrations of free IBA approach the levels of free IAA in a number of plants (Ludwig-Muller, 2000). IBA, like IAA, is also found in conjugated forms, yet at significantly lower levels than IAA (Ludwig-Muller et al., 1993). IBA and IAA can be interconverted (Bartel et al., 2001), which has led to the suggestion that IBA may act as a precursor to IAA. Arabidopsis mutants whose roots have reduced sensitivity to growth inhibition by IBA but normal sensitivity to IAA have been isolated recently (Bartel et al., 2001), and many of these have defects in beta-oxidation, which is the pathway by which IBA is thought to be converted to IAA (Zolman et al., 2001a, 2001b). These findings support a role for IBA as an IAA precursor.

Other lines of evidence suggest that IBA might also act directly as an auxin, rather than solely being an auxin precursor. First, IBA is the preferred auxin for the induction of root formation because it is much more potent than IAA or synthetic auxins (Ludwig-Muller, 2000). Several studies have demonstrated that internal IBA levels, not IAA levels, increase and stay elevated during IBA-induced root formation (Nordstrom et al., 1991; van der Krieken et al., 1992). Finally, the occurrence of several IBA resistant, IAA-sensitive mutants that do not have defects in beta-oxidation also suggest that IBA could act directly and not necessarily through conversion to IAA (Poupart and Waddell, 2000; Zolman et al., 2000).

To understand the endogenous role of IBA and the defects in these IBA-insensitive Arabidopsis mutants, it is necessary to examine how IBA is transported and the relationship between transport and action of IBA. This question has been examined using several approaches in plants other than Arabidopsis. Early

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studies relied on bioassays to detect auxin movement through tissues. Such studies report a polar movement of IBA in a basipetal direction in stems with similar or slower rates of movements to those of IAA (Went and White, 1938; Leopold and Lam, 1961). Interpretation of these studies is hampered by differences in sensitivity of bioassays to IBA and IAA (Thimann, 1952) and the effects of application of high auxin concentrations on auxin movement (Parker and Briggs, 1990). Further evidence for basipetal transport of IBA can be found in the work of Yang and Davies (1999). These authors showed that apically applied IBA can stimulate elongation of subtending nodes, suggesting IBA is transported basipetally in intact pea plants, but with slower kinetics than that of IAA (Yang and Davies, 1999). Additional studies have examined the distribution of radiolabeled IBA after application of a rooting solution to the base of explants. In most cases, however, these studies were not designed to distinguish between movement of auxin in the plant's vascular system and polar auxin transport (for example, see Epstein and Lavee, 1984; Wiesman et al., 1988; van der Krieken et al., 1992; Epstein and Ackerman, 1993). In one notable exception, IBA polar transport was examined in excised citrus leaf midribs and found to be twice as high in the basipetal direction as in the acropetal direction (Epstein and Sagee, 1992).

There is one report in the literature comparing IBA and IAA transport in the inflorescence axis of Arabidopsis ecotype Landsberg *erecta* (Ludwig-Muller et al., 1995). Using a nonstandard assay, transport of both IAA and IBA in the inflorescence axis was found to occur mostly in the acropetal direction but with some basipetal transport of both auxins (Ludwig-Muller et al., 1995). The authors of this study suggested this acropetal auxin movement could occur through the transpiration stream, possibly because of water loss at wound sites where leaves and siliques were removed from the axes (Ludwig-Muller et al., 1995). This is the only report in the literature in which there is more acropetal IAA movement than basipetal IAA movement in the Arabidopsis inflorescence axis in either Landsberg *erecta* or other ecotypes (Okada et al., 1991; Bennett et al., 1995), leaving it difficult to conclude whether the methods used in that report (Ludwig-Muller et al., 1995) were measuring cell-to-cell polar transport of either IAA or IBA in the Arabidopsis inflorescence axis. To the best of our knowledge, polar IBA transport in roots or hypocotyls of Arabidopsis and its regulation by auxin transport inhibitors, such as naphthyphthalamic acid (NPA), has not been examined. These are the tissues that have a clear IBA response and an altered sensitivity to IBA in mutant plants.

The major goals of this study were to gain a more detailed understanding of IBA transport in Arabidopsis and to use this information to clarify the role of this auxin in plant growth and development. Auxin transport was examined in several different tissues to determine where, and in which direction, IBA is transported. Furthermore, the rate and quantity of IBA transport was examined, as well as how this transport is affected by IAA efflux inhibitors and mutations in genes encoding putative IAA transport proteins. In addition, we have compared the effects of IAA and IBA on elongation of hypocotyls because differences in transport of the two auxins are detected in this tissue. Together, these results present a detailed picture of IBA transport with insights into its physiological role and provide the necessary background to interpret IBA mutant phenotypes.

RESULTS

Survey of IBA Transport in Several Arabidopsis Organs

IAA polar transport occurs in several distinct pathways in Arabidopsis. In the hypocotyl and inflorescence axis, IAA moves in a single direction from the apex to the base (basipetal transport). In the root, there are two distinct polar transport pathways. The first flows from the base of the root to the root tip (acropetal transport) and the second flows from the root tip back toward the base (basipetal transport).

To detect auxin movements in seedlings, tritiated auxin was applied from a 1-mm-diameter agar cylinder placed on top of roots or hypocotyls grown on agar and followed by measuring the amount of radioactivity that was taken up and transported into a distant tissue. Auxin transport was determined for several tissues, in which either ^{3}H-IAA or ^{3}H-IBA was applied continuously for the duration of the assay. For hypocotyls, radioactive auxin was applied below the cotyledons to examine basipetal transport toward the hypocotyl base. For acropetal transport in the hypocotyl, radioactive auxin was applied at the root shoot junction, and its movement to the hypocotyl apex was quantified. Radioactive auxin was applied at the root tip to examine root basipetal transport (RBT) within the first 5 to 10 mm of the root. For root acropetal transports (RATs), tritiated auxin was applied at the root shoot junction, and transport of auxin to the root tip was measured.

Measurements of both ^{3}H-IAA and ^{3}H-IBA movements in Arabidopsis tissues are reported in Table I and Figure 1A. IBA, like IAA, is transported in the root in both polarities and in the basipetal direction in the hypocotyl. In both roots and hypocotyls, IBA is transported at greater levels than is IAA. The elevated movement of IBA could be due to either greater IBA uptake or elevated polar IBA transport, but we did not differentiate between these two possibilities.

In contrast, there was no detectable IBA transport above background levels in the inflorescence stem at 18, 24, or 36 h after application in either a basipetal or an acropetal manner (Table I; data not shown). To determine if low levels of IBA transport could be

Table I. *Transport of IBA and IAA in Arabidopsis seedlings*

Transport Assay	Control	NPA[a]
	pmoles transported[b]	
Root acropetal		
IAA	4.5 ± 0.4	3.3 ± 0.2[c]
IBA	11.4 ± 1.4	11.8 ± 1.4
Root basipetal		
IAA	2.7 ± 0.2	1.6 ± 0.1[c]
IBA	10.2 ± 0.7	10.6 ± 0.6
Inflorescence basipetal		
IAA	1.4 ± 0.1	0.04 ± 0.02[c]
IBA	0.03 ± 0.005	0.02 ± 0.002

[a] For root transport assays, NPA concentration was 100 μM and for inflorescence transport assays NPA concentration was 15 μM. [b] Average and SE of 14 to 86 seedlings from two to seven experiments. [c] $P < 0.05$ as determined by one-tailed Student's *t* test for control vs. NPA. For all IBA transport experiments, the control vs. NPA treatments had $P > 0.05$.

detected in the inflorescence axis, an additional pulse-chase assay was used because this assay can identify small local amounts of auxin movement. ^{3}H-IAA was transported in a single wave, and the transport was completely inhibited by addition of the transport inhibitor NPA, whereas no detectable ^{3}H-IBA transport was observed in the presence or absence of NPA (Fig. 2). These results suggest that IBA is not transported in the Arabidopsis inflorescence axis, although IBA transport is readily measurable in the hypocotyl and root.

Polarity of IBA Transport in Roots and Hypocotyls

To identify the polarity of auxin transport in the hypocotyl, a comparison of ^{3}H-IAA or ^{3}H-IBA movement after application at the hypocotyl base and apex was performed (Fig. 1A). Both IBA and IAA are transported basipetally from the tip to the base of the hypocotyl, whereas neither IBA nor IAA is transported acropetally at levels above background.

IAA is transported basipetally in the first 5 to 7 mm of the Arabidopsis root tip, whereas acropetal IAA transport occurs along the whole length of the root (Rashotte et al., 2000). To determine if IBA is transported similarly in roots, ^{3}H-IAA or ^{3}H-IBA was applied mid-root in an agar cylinder 10 mm back from the root tip, and the amount of transport was measured in both directions (Fig. 1B). Because this site of auxin application is behind the zone of basipetal auxin transport, IAA and IBA movement is predominantly in the acropetal direction.

An additional assay was performed that determined how far IBA traveled from the tip. Labeled auxin was applied to the root tip, and after 5 h, the radioactivity in several 2-mm segments from the root tip back toward the base was quantified (Fig. 3A). These results indicate that root basipetal auxin transport occurs over the same distance for both IBA and IAA and is confined to the apical 7 mm of the root tip.

Rates of IBA Transport

To compare the rates of IAA versus IBA movement, a pulse-chase method for measuring the rate of root acropetal auxin transport was developed. It is easier to measure the rate of acropetal transport than basi-

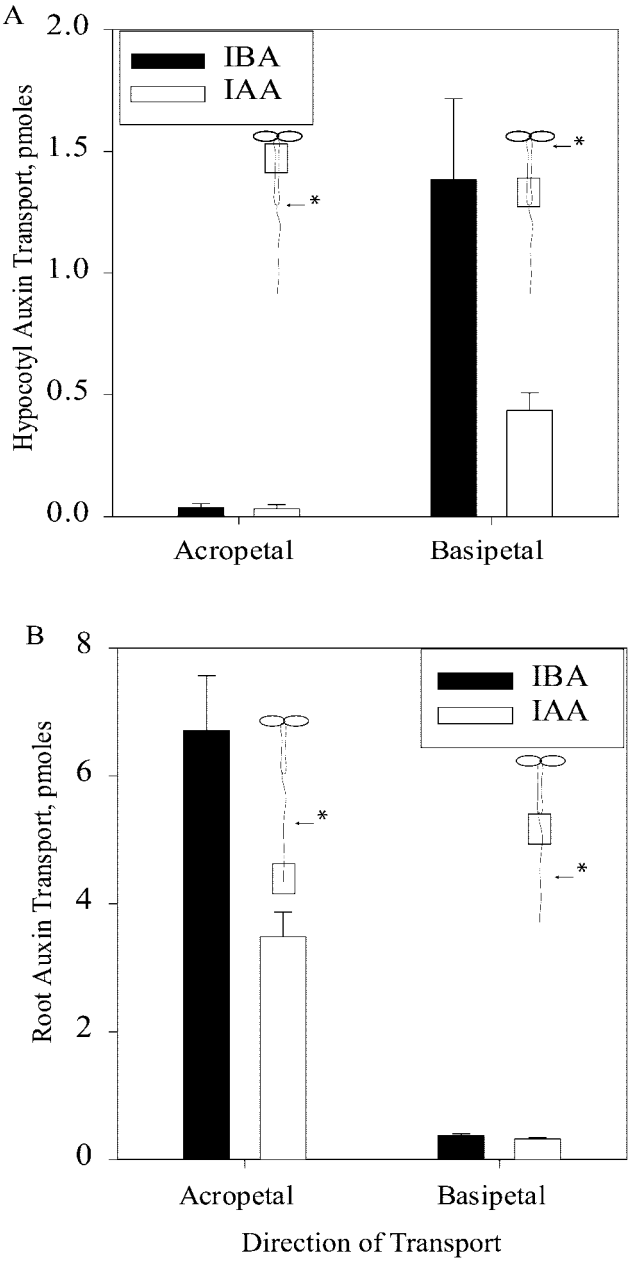


Figure 1. Polarity of IBA and IAA transport. A, Direction of IAA and IBA movement in hypocotyls was examined by applying ^{3}H-IBA or ^{3}H-IAA at the base or the tip of the hypocotyl and measuring radioactivity in distant 5-mm segments, as shown in inset (±SE; $n = 30$). B, Acropetal and basipetal movement of IBA and IAA in roots was examined by applying ^{3}H-IBA or ^{3}H-IAA at mid-root and measuring radioactivity in distant 3-mm segments, as shown in inset (±SE; $n = 10$). For the inset diagrams, the arrow and asterisk indicate the site of ^{3}H-IBA or ^{3}H-IAA application, and the boxes indicate the segments in which radioactivity was measured.

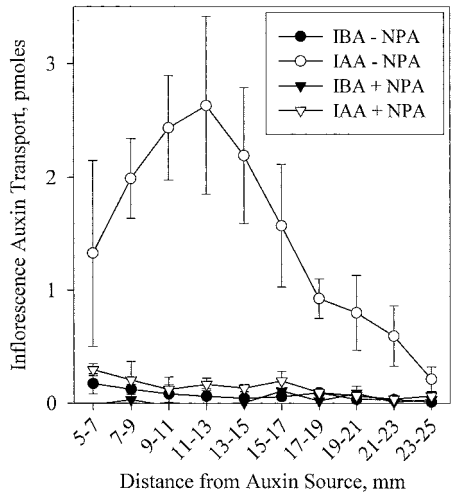


Figure 2. Inflorescence axis transport of IBA and IAA. IBA and IAA basipetal transport was examined in 25-mm inflorescence axis segments. The apical end of each segment was placed in ^{3}H-IBA or ^{3}H-IAA for 10 min, followed by a 90-min chase of the respective unlabeled auxin, either with or without NPA. The radioactivity in 2-mm segments was determined (±SE; $n = 3$).

petal transport because it occurs over a longer distance and with higher amounts of auxin movement. Using this assay, it was possible to identify the leading edge of auxin movement to estimate the rates of auxin movements (Fig. 3B). In 1 h, both auxins are transported the same distance to the segment 10 mm from the site of auxin application. Radioactivity levels in segments that are 12 mm or further from the site of labeled auxin application are at background levels. This experiment shows that IBA and IAA are transported at the same rate of 8 to 10 mm per hour.

Regulation of Auxin Transport by IAA Efflux Inhibitors

Polar IAA transport is reduced by inhibition of IAA efflux using inhibitors such as NPA and 2,3,5-triiodobenzoic acid (TIBA). These two inhibitors block efflux by binding to two different sites on the auxin efflux carrier complex, either a regulatory subunit or to the auxin-binding site, respectively (Rubery, 1990; Muday and DeLong, 2001). The mechanism of action of these compounds is not completely clear, but they may either directly block auxin movements or indirectly alter the cycling of auxin transport proteins to or from the plasma membrane (Muday and DeLong, 2001). The effect of NPA on IAA and IBA transport in roots and hypocotyls was determined (Tables I and II). There was no effect on the transport of IBA in any of these tissues, even with concentrations of NPA as high as 100 μM, which significantly reduce IAA transport. The ability of TIBA to block root acropetal IAA and IBA transport was also tested, and a concentration of 100 μM significantly reduced IAA movement (3.3 ± 0.2 pmol) as compared with untreated controls (4.5 ± 0.5 pmol), but did not significantly affect IBA movement (12.2 ± 1.4 pmol) as compared with controls (11.4 ± 1.4 pmol). These results indicate that IBA transport is not regulated by IAA efflux inhibitors and suggest that the inhibitor-sensitive auxin efflux carrier protein complexes that transport IAA differ from the protein complexes that transport IBA.

IBA Transport in Arabidopsis Mutants with Defects in Auxin Transport

To test the hypothesis that IBA is transported by different transport proteins than IAA, measurements of IBA and IAA transport were made in the *aux1* and *eir1* mutants, which have defects in genes predicted to encode IAA influx and efflux carriers, respectively (Parry et al., 2001b; Friml and Palme, 2002). *aux1* and *eir1* have been shown previously to have significant reductions in IAA accumulation and basipetal IAA transport in the root (Chen et al., 1998; Marchant et al., 1999; Rashotte et al., 2000, 2001). Levels of IBA and IAA transport in the roots of *aux1-7* and *eir1-1*

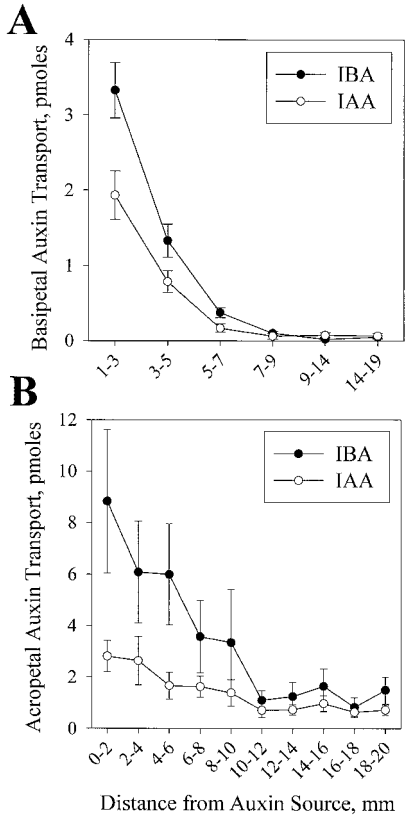


Figure 3. Distance and rate of IAA and IBA transport. A, Distance of IBA and IAA basipetal transport from the root tip was examined by applying ^{3}H-IBA or ^{3}H-IAA at the root tip and measuring radioactivity in either 2- or 5-mm segments spanning the indicated distance from the root tip. B, Rate of IBA and IAA acropetal transport was examined by applying ^{3}H-IBA or ^{3}H-IAA 20 mm from the root tip and measuring radioactivity in 2-mm segments at the indicated distance from the site of application (±SE; $n = 10$).

Table II. *Effect of light on hypocotyl basipetal transport of IBA and IAA*

Auxin Transported	Low Light		Darkness	
	Control	+NPA	Control	+NPA
	pmoles transported[a]			
IAA	1.4 ± 0.2	0.54 ± 0.09[b]	0.52 ± 0.04[b]	0.43 ± 0.06[b]
IBA	2.3 ± 0.3	2.2 ± 0.4	1.2 ± 0.1[b]	ND[c]

[a] Average and SE of 20 to 30 seedlings from two to three experiments. [b] Indicates values that were significantly different from low-light controls with $P < 0.001$ as determined by one-tailed Student's *t* test for control vs. 10^{-4} M NPA and by two-tailed Student's *t* test for low light vs. darkness. IAA transport in darkness was not significantly different in the presence and absence of NPA with a *P* value > 0.05. [c] N.D., Not determined

and in the wild-type Columbia background are shown in Table III. Basipetal IAA transport is significantly reduced in *aux1-7* and *eir1-1*, and acropetal IAA transport is reduced in *aux1-7*. We have found previously that acropetal IAA transport is unaffected by the *eir1-1* mutation (Rashotte et al., 2000). Basipetal IBA transport is similar to wild type in both *aux1-7* and *eir1-1*, and acropetal IBA transport is similar to wild type in *aux1-7*. These results suggest that IBA transport does not require the activity of either the EIR1 or AUX1 proteins, whereas IAA transport requires both of these proteins. Differences in values for basipetal transport in Table III, as compared with other values, reflect ecotype differences in transport and refinements in technique during the course of this work.

Examination of Transport Saturation

If IBA transport is protein mediated, then it should saturate at high auxin concentrations. Increasing concentrations of unlabeled IBA or IAA were added to an agar cylinder containing a constant level of radioactive auxin in an RBT assay (Fig. 4). Transport of IAA and IBA are both saturated at similar high concentrations of IAA and IBA, suggesting that IBA transport, like IAA transport, is carrier mediated.

Table III. *Transport of IBA and IAA in auxin transport mutants*

Transport	Columbia	*aux1-7*	*eir1-1*
	pmoles transported[a]		
Root basipetal			
IAA	1.1 ± 0.1	0.6 ± 0.03***	0.8 ± 0.05***
IBA	1.7 ± 0.08	1.7 ± 0.1	1.8 ± 0.1
Root acropetal			
IAA	3.7 ± 0.4	2.7 ± 0.2**	N.D.[b]
IBA	5.6 ± 0.6	5.6 ± 0.6	N.D.[b]

[a] Average and SE of 23 to 30 seedlings from three experiments. The *P* values were obtained by two-tailed Student's *t* test for Columbia vs. mutant plants. **, $P < 0.01$; ***, $P < 0.001$. No significant differences in IBA transport were detected for these mutants. [b] N.D., Not determined.

Relationship between Physiological Effects of IBA and Transport

Previous studies have examined the IBA and IAA sensitivity of Arabidopsis developmental processes, such as root elongation and lateral root formation (Poupart and Waddell, 2000; Zolman et al., 2000), but have not examined the response of hypocotyls to these two auxins. We examined the sensitivity of hypocotyl elongation to IBA and IAA using hypocotyls grown in dark, low, or high light. Figure 5 shows that IBA, but not IAA, is able to stimulate hypocotyl elongation significantly in high-light conditions, at concentrations ranging from 1 to 10 μM, with a maximum of about 50% stimulation at a concentration of 3 μM IBA. In all light conditions, hypocotyl elongation is more sensitive to inhibition by exogenous IAA than to IBA. This can be seen in dark and low-light conditions at concentrations ranging from 0.3 to 3 μM and in high-light conditions at concentrations ranging from 10 to 100 μM. These experiments also show that about 30-fold higher concentrations of auxin are required to inhibit hypocotyl elongation in high-light conditions relative to low-light or dark conditions.

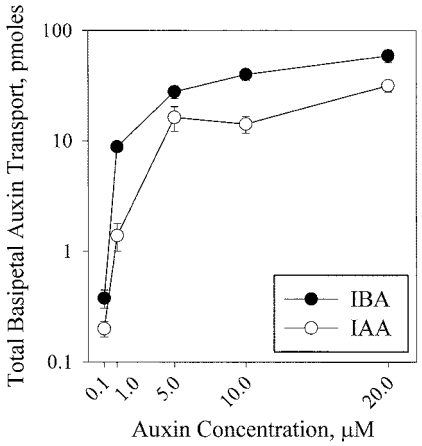
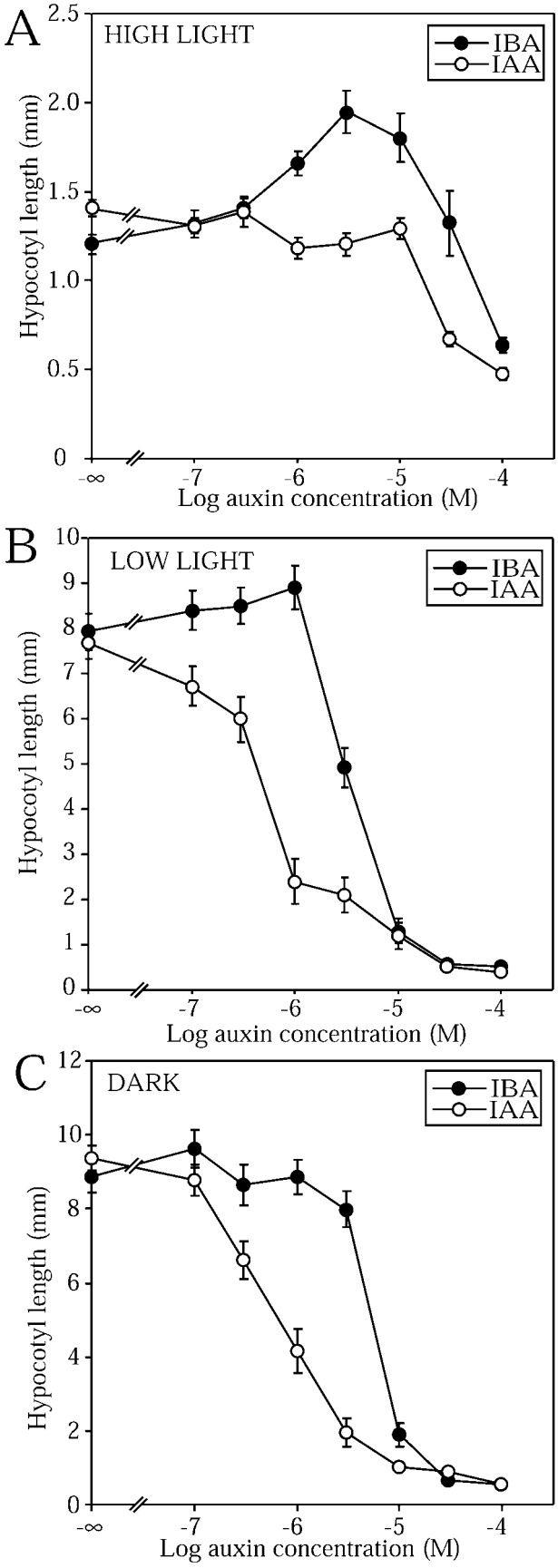


Figure 4. Saturation of auxin transport. The ability of IBA and IAA to saturate RBT was measured as a function of the movement of a constant amount of ^{3}H-IBA or ^{3}H-IAA with increasing unlabeled amounts of the respective auxin in distant 5-mm segments (±SE; $n = 10$).



Consistent with the differences between IBA and IAA on growth in hypocotyls under different light conditions are differences in hypocotyl IBA and IAA transport under similar conditions. The amount of IBA and IAA transport in hypocotyls under low light and in the dark are shown in Table II. In low light, there is more IBA transport than IAA transport, and only IAA transport is NPA sensitive. In the dark, both IAA and IBA transport are reduced about 2-fold (Table II), and the IAA transport is no longer sensitive to NPA.

DISCUSSION

The major goal of this work was to determine if the natural auxin, IBA, is transported in Arabidopsis with similar polarity, rate, and regulatory properties as is IAA. The effects of IAA and IBA on hypocotyl elongation were also examined to explore the relationship between polar transport and action of these two natural auxins. Polar transport of IBA was found to occur in hypocotyls and roots of Arabidopsis seedlings. IBA transport in hypocotyls occurred in a basipetal direction, with no detectable acropetal movement. In the roots, IBA transport occurred acropetally from the root shoot junction to the root tip at a rate of 8 to 10 mm h^{-1} and basipetally for a short distance back from the root tip. These results indicate that IBA transport mirrors the directional transport of IAA found in Arabidopsis seedlings (Rashotte et al., 2000, 2001), which was first demonstrated over 30 years ago in *Phaseolus coccineus* (Davies and Mitchell, 1972).

In stark contrast to the results with hypocotyls and roots, no IBA transport was detected in the inflorescence axis of Arabidopsis. Numerous experiments were performed to try to detect IBA transport in this tissue because there is one previous report of IBA movement in the inflorescence axis of the Landsberg *erecta* ecotype (Ludwig-Muller et al., 1995). In that paper, IAA and IBA were reported to have a predominantly acropetal movement in the inflorescence axis but were also found to move in a basipetal direction with both auxins transported at similar rates (Ludwig-Muller et al., 1995). These results conflict with other published papers on IAA movement in the inflorescence. Other studies have shown that IAA moves solely in the basipetal direction in the inflorescence and have confirmed that this movement is polar transport by using efflux inhibitors (Okada et al., 1991; Bennett et al., 1995). The acropetal transport observed by Ludwig-Muller et al. (1995) was suggested to be movement in the transpiration stream, and it is unclear whether the basipetal auxin

Figure 5. Effect of auxins on hypocotyl elongation. Dose response curves for hypocotyl elongation in response to 5 d on IAA or IBA under high white light (90 μmol m^{-2} s^{-1}; A), low light (5 μmol m^{-2} s^{-1}; B), or dark (C) conditions are reported (±SE; n = 6–26).

movements reported in that work were truly polar transport.

To look for IBA transport in our system, we performed several assays for time periods spanning from 5 to 36 h, with a range of IBA concentrations, and with tissue segments from different positions in the inflorescence axis from the Nossen ecotype and from plants of different ages. IBA transport was not detected in any of these experiments (J. Poupart and C.S. Waddell, data not shown). The pulse-chase experiment reported here examined IBA and IAA movement in the inflorescence axis by dividing this tissue into a number of small segments. Even when IBA transport was examined within 5 mm from the site of IBA application, no ^{3}H-IBA was detected above background levels. It remains a formal possibility that ^{3}H-IBA moves differently in the inflorescence of Landsberg *erecta*, which we did not specifically test in our experiments, although no ecotypic differences in inflorescence auxin transport have been reported previously.

To determine if the same auxin carrier protein complexes might mediate both IAA and IBA transport, auxin transport was examined in plants with mutations in genes believed to encode IAA transport proteins and in the presence of IAA efflux inhibitors. IBA and IAA transport were measured in roots of *eir1-1* and *aux1-7*, plants with mutations in genes predicted to encode IAA efflux and influx proteins, respectively (Parry et al., 2001b; Friml and Palme, 2002). There was no reduction or alteration in the transport of IBA in either the *eir1-1* or *aux1-7* mutant background, whereas basipetal IAA transport was significantly reduced in both mutants, and acropetal IAA transport was reduced in *aux1-7*. These results suggest that IBA is not transported by proteins encoded by the allelic *AGR1/EIR1/PIN2/WAV6* gene or the *AUX1* gene. Because the influx and efflux proteins are members of large gene families (Parry et al., 2001b; Friml and Palme, 2002), it is possible that other members of these gene families mediate IBA transport. Alternatively, IBA transport might be mediated by other proteins such as the AtMDR and AtPGP proteins, which have been implicated recently in IAA transport (Noh et al., 2001).

In experiments using the IAA transport inhibitors NPA and TIBA, concentrations as high as 100 μM had no effect on IBA transport in any tissues examined, whereas there was a significant reduction in IAA transport in the same tissues. This result suggests that IAA efflux carrier protein complexes sensitive to these inhibitors are unlikely to transport IBA. In the root and hypocotyl, in contrast to the inflorescence axis, polar IAA transport is not completely inhibited by these IAA efflux inhibitors. The residual level of IAA transport in these tissues may be mediated by auxin efflux carrier complexes that are insensitive to inhibitors, and these complexes may also mediate transport of IBA. Two additional lines of experimentation support this hypothesis. In roots of the *eir1-1* mutant, basipetal IAA transport is reduced, and the remaining transport of IAA is insensitive to NPA (Rashotte et al., 2000), consistent with the presence of a remaining NPA-insensitive carrier. This putative NPA-insensitive efflux carrier complex does not appear to act in the inflorescence axis; in this tissue, NPA treatment results in the almost complete inhibition of IAA transport. The absence of this putative NPA-insensitive efflux carrier complex in the inflorescence correlates with the absence of IBA transport.

One formal possibility to explain the lack of effect of IAA efflux inhibitors and mutations in putative IAA transport proteins is that IBA transport is not protein mediated. However, the strict polarity and tissue specificity of IBA movement argue against this possibility. Ludwig-Muller (1995) reported that IBA uptake is saturable for young Arabidopsis seedlings grown in culture. We also asked whether IBA movement is saturable in our transport assays. ^{3}H-IBA and ^{3}H-IAA movement were examined in the presence of increasing amounts of unlabeled IBA or IAA, respectively. The transport of IBA and IAA saturated at high concentrations, in a manner consistent with IBA and IAA transport being protein mediated. This saturation result, combined with the absence of IBA movement in the inflorescence axis and the strict directionality of IBA movement in other tissues, suggest that IBA transport is protein mediated but by proteins with different tissue specificity and regulatory properties than IAA carriers.

The levels of radioactive IBA transported in roots and hypocotyls are in general 2 to 4 times greater than those of IAA. The higher amounts of IBA transport could be due to either a greater uptake of IBA or more transport of IBA after it has been taken up. It is difficult to experimentally resolve these possibilities because we measured the radioactive auxin at a distance and did not measure the radioactivity at the site of application in these tissues. In contrast, in experiments with the inflorescence axis, IBA and IAA uptake into the first segment was compared. Even though IBA is not transported in this tissue, higher levels of tritiated IBA were found in the segment in direct contact with the solution containing radioactive auxin (data not shown). This suggests higher IBA uptake, although it could also reflect the absence of movement of IBA out of this tissue. The pulse-chase experiment in Arabidopsis roots, shown in Figure 3B, also suggests that IBA uptake is higher than IAA uptake. However, the higher amounts of IBA may result from an increased capacity for IBA transport. Without a compound that can effectively inhibit the transport of both IAA and IBA, we are unable to distinguish between higher uptake and higher transport capacity. Therefore, we are unable to resolve whether there is a greater total flux of IBA movement or whether the initial uptake of IBA is greater than

for IAA and the subsequent higher levels of transport represent a larger initial pool for transport.

We considered the possibility that applied IBA was converted to IAA before transport. The radiolabeled versions of IAA and IBA used in our experiments are labeled on the indole ring; therefore, interconversion of these auxins will not affect the associated radioactivity. Several lines of evidence argue against this possibility. First, the complete absence of IBA transport in the inflorescence axis is inconsistent with conversion of IBA into IAA, at least in this tissue, because some transport should be detectable if IBA is converted to IAA. In both roots and hypocotyls, IAA transport is inhibited by the efflux inhibitors, NPA and TIBA. The complete absence of inhibition of polar IBA transport by these inhibitors suggests that significant quantities of ^{3}H-IBA are not converted to ^{3}H-IAA during these assays either. Finally, we failed to detect conversion of IBA to IAA in seedlings. Experiments were performed in which Arabidopsis seedlings were incubated with ^{3}H-IBA for time periods from 5 min to 24 h, and the labeled metabolites were extracted and then separated by thin-layer chromatography (TLC; for details, see "Materials and Methods"). No free IAA was detected in any of the assays. We estimate that free IAA must be present in levels equal to 5% to 10% of IBA levels to be detected in these assays.

The phenotypes of a number of mutants need to be examined in the context of these results. Analysis of the auxin resistant *axr* and *aux* mutants of Arabidopsis, which are resistant to root growth inhibition by IAA, has helped dissect the mode of action and transport of this auxin in plants (Leyser, 1997; Muday, 2001; Kepinski and Leyser, 2002). By analogy, mutants specifically resistant to IBA, but retaining wild-type sensitivity to IAA also have been identified and analyzed to help dissect the role of IBA in plant growth and development (Poupart and Waddell, 2000; Zolman et al., 2000). A subset of these mutants are resistant to the synthetic auxin 2,4-dichlorophenoxyacetic acid and to the auxin transport inhibitors NPA, TIBA, and 9-hydroxyfluorene-9-carboxylic acid. It is difficult to reconcile the inhibitor resistance phenotype of these mutants with the fact that IBA transport is not sensitive to such inhibitors. Many mutants that are insensitive to IAA exhibit reduced sensitivity to auxin transport inhibitors (Muday et al., 1995). The IBA-insensitive mutants are normally sensitive to IAA; therefore, in response to NPA treatment, local IAA accumulation at the root tip may cause an increase in IAA conversion to IBA, to which the roots are insensitive. The local accumulation of IAA after such IAA efflux inhibitor treatments has been reported (Casimiro et al., 2001), although the accumulation of IBA in response to elevated IAA levels under these conditions has not been investigated.

Previous reports suggest that IBA is not a substrate for the EIR1 protein because differential root growth in the *eir1* mutant can be stimulated by IAA but not by IBA (Poupart and Waddell, 2000; Zolman et al., 2000). This result is consistent with the transport experiments reported here. A similar conclusion for the role of AUX1 in mediating IBA transport is not as clear. In this study, we find no role for AUX1 in mediating IBA transport, yet two previous lines of experimentation have suggested IAA and IBA uptake may occur by similar mechanisms. The first is the ability of excess IAA to prevent labeled IBA uptake (Ludwig-Muller et al., 1995). These results can be reconciled by the hypothesis that IAA, but not IBA, is transported into the cell through the AUX1 carrier, whereas both IAA and IBA are transported into the cell through an alternative influx carrier, which is the only mode for IBA entry into the cell. If this was the case, then excess IAA would compete with labeled IBA uptake to the cell as reported (Ludwig-Muller et al., 1995). The second line of experimental evidence supporting a role for AUX1 mediation of IBA influx is the report that the *aux1-7* mutant is reduced in root growth inhibition by IBA (Zolman et al., 2000). However, the reduced growth inhibition is modest; *aux1-7* root growth is reduced 35% in the presence of IBA relative to untreated plants as compared with approximately 55% growth inhibition for wild-type plants. Furthermore, the *aux1-7* mutant responds in a wild-type manner to IBA induction of lateral roots. The reduced sensitivity of *aux1-7* to growth inhibition by IBA, therefore, may be the indirect result of excess conversion of IBA to IAA that then affects root growth (Bartel et al., 2001). Overall, these data suggest that if *AUX1* plays a role in IBA transport, it is an indirect one.

The tissue specificity of IBA transport reported here supports the possibility that this endogenous auxin plays a role in growth and development of some Arabidopsis tissues. Several previous studies have shown that IBA, like IAA, inhibits root elongation and induces lateral root formation (Poupart and Waddell, 2000; Zolman et al., 2001b). IBA affects stem elongation in pea seedlings (Yang and Davies, 1999), but its effect on Arabidopsis hypocotyl elongation has not been examined previously. Therefore, we examined the growth sensitivity of Arabidopsis hypocotyls to IAA and IBA. Hypocotyls were sensitive to growth stimulation by low concentrations of IBA in high-light conditions, but insensitive to growth stimulation if grown in low light or dark. In contrast, hypocotyls were insensitive to growth stimulation by IAA at any concentration tested or under any light condition. Interestingly, in pea epicotyls, both auxins can stimulate growth at low concentrations, and the growth-promoting effect moves in a basipetal polarity, consistent with the data reported here for IBA and IAA polar transport in hypocotyls (Yang and Davies, 1999). Both dark- and light-grown Arabidop-

sis hypocotyls were sensitive to growth inhibition by both auxins but were more sensitive to IAA in this assay.

The amounts of auxin transport in the hypocotyl change between low-light and dark conditions. In the dark, IAA transport and IBA transport are both reduced relative to low-light-grown hypocotyls, and IAA transport is no longer NPA insensitive, suggesting that similar mechanisms may control both IBA and IAA transport in the dark. Previous reports on hypocotyl growth are consistent with auxin transport in the dark being mediated by an IAA efflux carrier inhibitor-insensitive mechanism (Jensen et al., 1998). The interactions between light and auxin signaling are only now becoming apparent (Tian and Reed, 2001; Swarup et al., 2002). There is a complex interaction between light and auxin levels, transport, and hypocotyl elongation. Low concentrations of exogenous IBA stimulate hypocotyl elongation only in light-grown seedlings. Transgenic or mutant Arabidopsis plants with altered IAA levels show altered hypocotyl growth only in light-grown seedlings (Boerjan et al., 1995; Romano et al., 1995). These results suggest that light-grown hypocotyls are more sensitive to growth stimulation by auxin. There are much lower levels of IAA in dark-grown hypocotyls and roots as compared with light-grown plants (Bhalerao et al., 2002), indicating that light also controls the level of auxin synthesis. Finally, light-grown but not dark-grown hypocotyls show growth inhibition by NPA (Jensen et al., 1998) and NPA regulation of IAA transport (this report). Therefore, light influences auxin synthesis, transport, and response.

The physiological significance of the absence of IBA transport in the inflorescence axis should also be considered. There are no reported measurements of IBA levels in the inflorescence, although this tissue is an abundant source of IAA (Brown et al., 2001) and is a site of conversion between IAA and IBA (Ludwig-Muller and Epstein, 1994). In addition, the mutants that have been isolated with altered IBA sensitivity largely have no apparent inflorescence phenotypes (Bartel et al., 2001). Two exceptions to this statement are the *pxa1* and *aim1* mutants, which have defects in fatty acid mobilization (Richmond and Bleecker, 1999; Zolman et al., 2001b). The inflorescences of *pxa1*, like all parts of this plant, are reduced in size, perhaps as a side effect of the fatty acid utilization, not IBA insensitivity (Zolman et al., 2001b). The inflorescence defects are much more striking in the *aim1* mutant, which has a defect in beta-oxidation of both lipids and auxins. This mutant is resistant to root growth inhibition by IBA (Zolman et al., 2000), so the inflorescence defects could be a result of altered IBA metabolism or altered lipid metabolism, although this has not been experimentally tested (Richmond and Bleecker, 1999). Given our finding that IBA is not transported in the inflorescence axis, any direct role that IBA has in the phenotype of these mutants must occur through local synthesis.

In summary, our study has revealed the basic outline of IBA transport within Arabidopsis, in terms of polarity, tissue specificity, distance, and rate. Our results suggest that different IAA efflux carrier protein complexes may mediate IAA and IBA transport. The best characterized IAA transport proteins, AUX1 and EIR1, do not have a role in IBA transport. Several of our results suggest the presence of an uncharacterized auxin efflux carrier complex, which is insensitive to NPA and transports both IAA and IBA. This study provides the groundwork necessary for understanding the differences and similarities between polar transport of IAA and IBA; this, in turn, will be critical for understanding the role of IBA in plant growth and development and in characterization of the recently isolated mutants with altered sensitivity to IBA.

MATERIALS AND METHODS

Chemicals

Chemicals were purchased from the following suppliers: NPA from Chemical Services (West Chester, PA), absolute ethanol from McCormick Distilling Co., Inc. (Weston, MO), and 3-[5(n)-^{3}H]-IAA (27 and 25 Ci mmol^{-1}) from Amersham (Arlington Heights, IL). 3-[^{3}H(G)]-IBA (25 Ci mmol^{-1}) was prepared in a custom synthesis under conditions designed to label the indole ring by American Radiolabeled Chemicals (St. Louis). All other chemicals were obtained from Sigma (St. Louis).

Seed Germination and Plant Growth

Wild-type Arabidopsis seeds (ecotype Columbia) and *aux1-7* and *eir1-1* seeds were obtained from the Arabidopsis Biological Resource Center (Ohio State, Columbus). All experiments were performed with ecotype Nossen-0, except where indicated. Seeds were soaked in distilled water for 30 min and surface sterilized with 95% (v/v) ethanol for 5 min and 20% (v/v) bleach with 0.01% (v/v) Triton X-100 for 5 min. After five washes in sterile distilled water, seeds were germinated and grown on 9-cm petri plates containing sterile control medium containing 0.8% (w/v) agar (Sigma type M, plant tissue culture), 1× Murashige and Skoog salts (pH 6.0), 1.5% (w/v) Suc, 1 μg mL^{-1} thiamine, 1 μg mL^{-1} pyridoxine HCl, and 0.5 μg mL^{-1} nicotinic acid. Seeds were grown in vertically oriented petri dishes in continuous 90 μmol m^{-2} s^{-1} fluorescent light at room temperature (22°C) for root auxin transport experiments. Seedlings used in hypocotyl assays were grown in horizontally oriented petri dishes at room temperature (22°C) but exposed to only 5 μmol m^{-2} s^{-1} of constant fluorescent light to increase hypocotyl length. Plants for continuous pulse inflorescence axis assays were grown on a 1:1:1 (w/w) mixture of perlite:vermiculite:Sunshine mix number 1 (Sun Gro Horticulture Inc., Bellevue WA). Plants were grown at 24°C under continuous white fluorescent light and fertilized twice during their growth period with 0.25× Hoagland solution. Light intensity was approximately 90 μmol m^{-2} s^{-1}. Plants grown for pulse-chase inflorescence axis assays were grown in metro mix 220 soil at room temperature (22°C) at 90 μmol m^{-2} s^{-1} fluorescent light on a 16-h-light: 8-h-dark cycle for 25 d.

Inflorescence Auxin Transport Assays

Inflorescence axis transport measurements were conducted on 25-d-old plants using a continuous pulse of radioactive auxin as described previously (Okada et al., 1991; Brown et al., 2001). In this assay, 100 nM ^{3}H-IAA or ^{3}H-IBA were applied to a 2.0-cm inflorescence axis segment in the presence or absence of 15 μM NPA, and transport into the basal 5 mm of that segment was measured after 18 h. Equivalent amounts of solvent only (dimethyl sulfoxide [DMSO]) were added to assays without NPA. Each segment was

placed into 2.5 mL of scintillation fluid overnight, and the amount of radioactivity within each sample was determined using a Beckman LS6500 scintillation counter (Beckman Instruments, Fullerton, CA) for 2 min.

Inflorescence axis transport was also measured using a pulse-chase experiment (modified from Parry et al., 2001a). This procedure is similar to the continuous pulse experiment above except that 400 nM ^{3}H-IAA or ^{3}H-IBA was applied to a 2.5-cm inflorescence axis segment for 10 min, then briefly rinsed and placed in a solution of nonradioactive auxin of similar concentration for 90 min. Higher concentrations of radioactive auxins were used in the pulse chase experiments because plants are in contact with radioactivity for very short periods, and higher levels of radioactivity are necessary to get sufficient counts in the segments. Transport was measured 100 min after the experiment started in the basal-most 10 2-mm segments as above.

Hypocotyl Transport Assay

Hypocotyl transport measurements were obtained for 5-d-old seedlings grown under low light or in the dark. Seedlings were transferred to control plates and oriented vertically along the surface of the agar. In experiments to examine hypocotyl basipetal transport, seedlings were aligned by their shoot apical meristems, and cotyledons were excised immediately preceding the experiment, leaving approximately 10 mm of hypocotyl. In experiments to examine acropetal transport, the root shoot junctions were aligned, and no tissues were removed. There was very little growth in a 5-h experiment. In these assays, mixtures containing 1% (w/v) agar, 100 nM ^{3}H-IAA, or ^{3}H-IBA with either 100 μM NPA or DMSO at the same concentration (1% [v/v]) were prepared in 3-mL scintillation vials. A narrow stem transfer pipette was carefully inserted into the hardened agar mixture such that a long 1-mm diameter cylinder of agar was removed. This cylinder containing radioactive auxin mixture was applied such that the agar was in contact with the cut surface of the hypocotyl for hypocotyl basipetal transport and on top of the seedling, just above the root shoot junction for hypocotyl acropetal transport. Plates remained vertically oriented in the dark to avoid auxin degradation (Stasinopoulos and Hangarter, 1989). Radioactive auxin transport was measured after 5 h by scintillation counting of a 5-mm segment of hypocotyl from the opposite end of the hypocotyl. The distance the auxin was transported was approximately 10 mm for dark- and low-light-grown seedlings from the cylinder of applied radioactive auxin. For experiments with dark-grown hypocotyls, all manipulations were performed with the aid of a green safelight.

Root Transport Assays

Basic root auxin transport measurements were made on 6- or 7-d-old vertically grown seedlings as in Rashotte et al. (2001), which is a modification of the original protocol developed in Rashotte et al. (2000). In all root transport assays, seedlings were transferred to control plates and oriented vertically such that the site where radioactive auxin would be applied was aligned. In each of these assays, mixtures containing 1% (w/v) agar, 100 nM ^{3}H-IAA, or ^{3}H-IBA with either 100 μM NPA, TIBA, or 1% (v/v) DMSO were prepared in 3-mL scintillation vials and prepared and applied as above. Standard placement of radioactive agar cylinders was so there was just contact with the root tips for RBT and on top of the seedlings, just on the root side below the root shoot junction for RAT. Auxin transport was measured after 5 h for RBT by first removing the 1 mm of tissue in contact with the agar cylinder, then cutting 2- or 5-mm segments (as indicated) from the site of application along the desired length. In RAT, measurements were made either after 18 h from an application site at the root shoot junction using a 5-mm segment at the root tip, which was approximately 15 to 20 mm from the site of auxin application. The amount of radioactivity in each segment was determined as described above.

For the experiments to determine polarity of auxin transport in the root (Fig. 2B), RBT and RAT were measured for each root. Radioactive agar cylinders, as described above, were placed 10 mm back from the root tip in this experiment, and transport occurred during a 5-h assay. RBT was determined by measurement of radioactivity in a 3-mm segment at the root base, which was approximately 7 mm from the site of application. RAT was quantified by determination of the radioactivity in a 3-mm segment at the root tip, which measured auxin movement 7 mm from the site of application.

For RAT pulse-chase experiments (Fig. 3B), seedlings were placed on agar plates with the root shoot junction aligned, and a cylinder containing 400 nM ^{3}H-IBA or ^{3}H-IAA was applied 20 mm from the root tip. After 10 min, the radioactive agar cylinder was removed from the seedlings, and all seedlings were moved to a new agar plate where a nonradioactive agar cylinder of similar auxin concentration was applied in the same position on the seedling for a 50-min chase. Ten 2-mm segments were excised starting from the root tip and analyzed as above.

RBT assays to determine saturation kinetics were conducted as basic continuous pulse assays with a constant level of ^{3}H-IBA or ^{3}H-IAA and increasing amounts of unlabeled IBA or IAA from 0.1 to 20.0 μM, as indicted in Figure 4, in each agar cylinder. The amount of DMSO used as an auxin solvent was maintained at 0.1% (v/v) of the final concentration of each agar cylinder. A single 5-mm segment back from the root tip, excluding the 1 mm of root tip in contact with the agar cylinder, was collected and counted after 5 h as above.

Analysis of the Sensitivity of Hypocotyl Elongation to IBA and IAA

Seeds were surface sterilized using the vapor phase sterilization protocol (Clough and Bent, 1998). Seeds were placed in open microfuge tubes in a desiccating jar. One hundred milliliters of a 10% (v/v) sodium hypochlorite solution (commercial bleach) were placed in a 250-mL beaker in the jar with the seed in a fume hood. Three milliliters of concentrated hydrochloric acid was added to the bleach, and the desiccating jar was quickly closed. Seeds were left to sterilize for three to 6 h, after which the jar was opened carefully in a fume hood, the tubes were removed from the jar, and sterile water was added to each tube of seeds. Seeds were stratified 4 to 7 d in the dark at 4°C before being germinated. For growth analyses only, IAA and IBA were dissolved in 1 mL of 1 N NaOH and diluted with 49 milliliters of deionized water to a final stock concentration of 1 mg mL^{-1} and filter sterilized. These stocks had pH values of 11.5 and 11.3 for IBA and IAA, respectively. Appropriate amounts of the sterile stocks were added to media after autoclaving to obtain the different concentrations required. Because the growth media (GM) used in these studies is buffered (see below), addition of the stocks did not result in any pH change in the media.

The effects of auxins present in horizontally oriented GM plates on hypocotyl elongation were investigated. GM medium containing 0.8% (w/v) Difco agar was used instead of solidified nutrient solution. GM medium consists of 1× Murashige and Skoog basal salts, 1% (w/v) Suc, 0.5 g L^{-1} MES, 1 mg of thiamine, 0.5 mg L^{-1} pyridoxin, 0.5 mg L^{-1} nicotinic acid, and 100 mg L^{-1} myo-inositol, with pH adjusted to 5.7 with 1 N KOH (Valvekens et al., 1988). After stratification, seeds plated directly on auxin-containing plates, or control media were placed either in dark, high constant white light conditions (90 μmol m^{-2} s^{-1}) or low-light conditions (5 μmol m^{-2} s^{-1}). Hypocotyl length was determined on 5-d-old seedlings by tracing magnified seedlings (approximately 5-fold) using an overhead projector. A transparent ruler placed beside the hypocotyls was also traced for use as a scale bar. The tracings were then digitally scanned, and measured using the public domain National Institutes of Health Image program (http://rsb.info.nih.gov/nih-image/). Similar results were obtained in three separate trials for each light condition. Data from a single representative trial are presented.

Analysis of IBA Metabolism

Analysis of ^{3}H-IBA metabolism was performed using a protocol adapted from Delarue et al. (1999). Twenty-five 8-d-old seedlings were incubated in a 35-mm petri dish containing 2 mL of liquid GM media and 1 μCi of ^{3}H-IBA for periods of time ranging from 5 min to 24 h. Seedlings were removed from the incubation medium, rinsed twice with 2 mL of sterile distilled water, and blotted dry. The seedlings were transferred to a new microfuge tube containing 200 μL of methanol, crushed using a small plastic pestle (Kimble, Vineland, NJ), and left to extract overnight in methanol at 4°C. Extracts were centrifuged to clear debris, dried partially in a centrifugal evaporator (Speedvac, Savant Instruments, Holbrook, NY), and loaded onto silica gel TLC plates with aluminum backing (Merck, Darmstadt, Germany). Unlabeled IAA and IBA stocks were loaded in lanes at both sides of the plate and radioactive IAA and IBA were loaded in a control lane on one side of the plates. The mobile phase consisted of chloroform:methanol:water (84:

14:1 [v/v]; Piskornik and Bandurski, 1972) and resulted in well-separated IAA and IBA peaks (R_F values of 0.73 and 0.78, respectively). After migration, control lanes containing nonradioactive auxins were cut off the plate, sprayed with Ehmann's reagent (Ehmann, 1977), and heated to reveal the position of the IAA and IBA spots. The remaining plate was cut into individual lanes, and each lane was cut in 5-mm sections that were placed directly in scintillation vials containing 5 mL of scintillation cocktail. Pieces of TLC plate were allowed to extract overnight in the dark before radioactivity was measured using a scintillation counter. Free IBA was detected in the appropriate migration position. Radioactivity levels were never above background at the position of IAA migration, indicating that levels of conversion were below the detection limits of this assay.

Statistics

Statistical analyses of data were performed using Excel (Microsoft, Redmond, WA). Multiple experiments were analyzed simultaneously by comparing averages, using each root as an independent sample. The data were analyzed by a one-tailed Student's *t* test for equal variance for transport inhibitor treatments and by a two-tailed Student's *t* test for equal variance when comparing the wild type with mutant or inhibitor treated seedlings or to compare IAA and IBA treatments in physiological assays. The *P* values are reported.

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CORRECTION

The authors of the articles listed below discovered an error in a spreadsheet developed by their laboratory. This error resulted in an incorrect conversion of radioactive counts to the appropriate molar units. The absence of a 1,000-fold difference led them to report their auxin transport values as pmol instead of reporting them correctly as fmol. The following articles were affected by this conversion error.

Vol. 122: 481–490

Rashotte A.M., Brady S.R., Reed R.C., Ante S.J., and Muday G.K. Basipetal Auxin Transport Is Required for Gravitropism in Roots of Arabidopsis.

The units of radioactive indole-3-acetic acid transport were mislabeled in Figure 3 and Tables II and IV, as were the units of radiolabeled benzoic acid in Tables II and IV. Both control and experimental values should have been reported as fmol rather than pmol. This change in units does not alter the interpretation of the data since all experiments had internal controls.

Vol. 133: 761–772

Rashotte A.M., Poupart J., Waddell C.S., and Muday G.K. Transport of the Two Natural Auxins, Indole-3-Butyric Acid and Indole-3-Acetic Acid, in Arabidopsis.

The units of radioactive indole-3-acetic acid and indole-3-butyric acid transport were mislabeled in Figures 1 to 4 and Tables I to III and in the text on page 764. Both control and experimental values should have been reported as fmol rather than pmol. This change in units does not alter the interpretation of the data since all experiments had internal controls. The one exception to this change is the inflorescence transport data reported in Table I, which are correctly reported as pmol and are consistent with greater amounts of indole-3-acetic acid transport in the inflorescence. Additionally, the higher number of counts in the inflorescence data in Table I is also due to the higher amounts of radioactive auxin added. The amount of added radioactivity in this continuous pulse of auxin in the inflorescence assay was incorrectly reported in the methods as 100 nM and should have been reported as 333 nM.

CORRECTION

Vol. 133: 761–772, 2003

Rashotte A.M., Poupart J., Waddell C.S., and Muday G.K. Transport of the Two Natural Auxins, Indole-3-Butyric Acid and Indole-3-Acetic Acid, in Arabidopsis.

Plant Physiology regrets that due to an editorial error, a journal name was listed incorrectly in the references for this article. The corrected references are listed below.

Clough SJ, Bent AF (1998) Floral dip: a simplified method for transformation of *Arabidopsis thaliana*. Plant J **16:** 735–743

Epstein E, Ackerman A (1993) Transport and metabolism of indole-3-butyric acid in cutting of *Leucadendron discolor*. Plant Growth Regul **12:** 17–22

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Planta (1985) 165:232–241

Planta

Gas chromatography-mass spectrometry evidence for several endogenous auxins in pea seedling organs

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Abstract. Qualitative analysis by gas chromatography-mass spectrometry (GC-MS) of the auxins present in the root, cotyledons and epicotyl of 3-d-old etiolated pea (*Pisum sativum* L., cv. Alaska) seedlings has shown that all three organs contain phenylacetic acid (PAA), 3-indoleacetic acid (IAA) and 4-chloro-3-indoleacetic acid (4Cl-IAA). In addition, 3-indolepropionic acid (IPA) was present in the root and 3-indolebutyric acid (IBA) was detected in both root and epicotyl. Phenylacetic acid, IAA and IPA were measured quantitatively in the three organs by GC-MS-single ion monitoring, using deuterated internal standards. Levels of IAA were found to range from 13 to 115 pmol g^{-1} FW, while amounts of PAA were considerably higher (347–451 pmol g^{-1} FW) and the level of IPA was quite low (5 pmol g^{-1} FW). On a molar basis the PAA:IAA ratio in the whole seedling was approx. 15:1.

Key words: Auxin in pea tissues – Cotyledon (auxins) – Epicotyl – *Pisum* (auxins) – Root auxins.

Introduction

For many years, it was assumed that 3-indoleacetic acid (IAA) was the only endogenous auxin in plants. Recently, however, evidence has been obtained using gas chromatography-mass spectrometry (GC-MS) for the presence of 3-indolepropionic acid (IPA) in squash hypocotyls (Segal and Wightman 1982) and for phenylacetic acid (PAA) in young leaves of tobacco (Wightman and Lichty 1982). 4-Chloro-3-indoleactic acid (4Cl-IAA) has been isolated from immature seeds of pea (Marumo et al. 1968; Engvild et al. 1978) and *Vicia* (Pless et al. 1984), and the methyl ester of this compound (4Cl-IAA-methyl ester) is present in seeds of *Lathyrus*, *Vicia* and *Pisum* (Engvild et al. 1980). Earlier chromatographic investigations indicated that 3-indolebutyric acid (IBA) may be an endogenous constituent of tobacco leaves (Bayer 1969) and potato peel (Blommaert 1954).

In the work described in the present paper, we have made a qualitative study, using GC-MS, of the auxins PAA, IAA, IPA, 4Cl-IAA and IBA and of the auxin metabolite, 4Cl-IAA-methyl ester, in the root, cotyledons and epicotyl of 3-d-old pea seedlings. We have also looked for potential precursors of IAA in roots, such as 3-indolepyruvic acid, tryptamine, 3-indoleacetonitrile and tryptophol, in order to gain some insight into the possible biosynthetic pathway for IAA in this organ. Phenylacetic acid, IAA and IPA have been measured quantitatively in each of the three seedling organs by GC-MS-single ion monitoring (GC-MS-SIM) using deuterated internal standards.

Abbreviations: IAA = 3-indoleacetic acid; 4Cl-IAA = 4-chloro-3-indoleacetic acid; IBA = 3-indolebutyric acid; IPA = 3-indolepropionic acid; PAA = phenylacetic acid; GC-MS = gas chromatography-mass spectrometry; HPLC = high-performance liquid chromatography; PFB = pentafluorobenzyl ester; PFBBr = pentafluorobenzyl bromide; SIM = single-ion monitoring; TMSI = trimethylsilyl ester

Material and methods

Plant material. Seeds of pea (*Pisum sativum* L., cv. Alaska; Rodger Brothers Seed Co., Idaho Falls, Id., USA) were soaked for 4 h in water, then planted in plastic pans (35·30·1 cm^3) containing approx. 8 cm of vermiculite, and covered with aluminium foil. The seedlings were grown in darkness in a growth chamber at 23° C for 72 h. They were then harvested, washed, separated into root, epicotyl and cotyledons, and weighed out into samples of 20–25 g FW. The tissue was immediately frozen with liquid nitrogen and held in a freezer at −15° C until it was analysed, normally within two weeks.

Extraction. Twenty-five grams (FW) of plant material were added to 100 ml of grinding medium. For acidic and neutral

Table 1. Retention times (in mins) of auxins, auxin precursors and metabolites in five HPLC solvent gradients. Composition and description of gradients are given in *Material and methods*

Compound	HPLC gradient				
	1	2	3	4	5
Phenylacetic acid	23 22	–	–	–	–
3-Indoleacetic acid	23 58	–	–	–	–
3-Indolepropionic acid	32 51	–	–	–	–
4-Chloro-3-indoleacetic acid	40 51	–	–	–	–
3-Indolebutyric acid	47 48	–	–	–	–
3-Indoleacetic acid methyl ester	–	12 49	–	–	43 17
3-Indolepropionic acid methyl ester	–	16 12	–	–	50 15
4-Chloro-3-indoleacetic acid methyl ester	–	17 48	–	30 50	–
3-Indolebutyric acid methyl ester	–	19 38	–	–	53 26
3-Indolepyruvic acid methyl ester	–	–	–	–	27 30
3-Indoleethanol (tryptophol)	–	–	23 21	–	–
3-Indoleacetonitrile	–	–	25 23	–	–

compounds, the grinding medium contained 80 ml methanol, 10 ml 1 M ammonium-acetate buffer, pH 6.5, 0.8 ml mercaptoethanol and 9.2 ml permanganate double-distilled water (R.O. Morris, Oregon State University, Corvallis, USA, personal communication). For the basic compound, tryptamine, the grinding medium contained 80 ml methanol and 20 ml 0.1 N HCl. The tissue was ground for 45 s in a Polytron homogeniser (Brinkmann Instrument Co., Rexdale, Ont., Canada). When required, deuterated samples of three authentic auxins were added to the homogenate, which was then stirred for 2 h using a Corning magnetic stirrer (CANLAB, Toronto, Canada) at 4° C in the dark. Cellular debris was removed by centrifugation and the methanol evaporated from the supernatant in vacuo in a rotary evaporator at 20° C. The samples were usually frozen overnight at this stage and any precipitate which remained after thawing was removed by centrifugation. The pH of the sample was adjusted to 7.5 with 1 N NaOH, and neutral compounds were obtained by three extractions with freshly distilled diethyl ether. Samples intended for analysis of acidic auxins were then extracted three times with ether at pH 3.0, while samples suspected of containing tryptamine were extracted three times with ether at pH 12. The ether extracts were concentrated to a small volume in vacuo in a rotary evaporator at 20° C and the samples were transferred to vials. The volume of the solution was further reduced to less than 0.5 ml under a gentle stream of nitrogen as 35° C.

In the experiment in which an attempt was made to demonstrate the presence of endogenous 3-indolepyruvic acid, the concentrated acid ether fraction of one sample of root tissue was methylated before high-pressure liquid chromatography.

High-pressure liquid chromatography (HPLC) was carried out using a Beckman-Altex system (Beckman Instruments, Montreal, Canada) consisting of two Model 100A pumps controlled by a Model 420 microprocessor and connected to an Alltech C_{18} column (25 cm long, 4.6 mm internal diameter; 10 µm particle size). The flow rate was 1 ml min^{-1} and an ultraviolet detector (Model 152) was used at 254 nm.

Neutral samples suspected of containing tryptophol or 3-indoleacetonitrile were dissolved in 2 ml 10% methanol and pre-purified by loading onto a SepPak C_{18} cartridge (Waters Assoc., Milford, Mass., USA), washing with 1 ml 20% methanol and eluting with 1.2 ml 80% methanol. Other neutral samples and samples containing acidic compounds were diluted to 1.1 ml with the starting solvent and filtered through a 0.45 µm Millipore filter (Millipore Ltd., Mississauga, Ontario, Canada. A 1-ml injection loop was used.

The following solvents were used:
Solvent A: water
Solvent B: methanol
Solvent C: 0.1 N formic acid in water
Solvent D: 0.1 N formic acid in methanol
Redistilled formic acid, water double-distilled in the presence of potassium permanganate, and HPLC-grade methanol were used to prepare these solvents. The following gradients were employed;

Gradient 1: 10–45% D in C over 10 min followed by isocratic elution with 45% D in C (used for crude acid ether fractions containing PAA, IAA, IPA, 4Cl-IAA or IBA).

Gradient 2: 50–100% B in A over 20 min (used for re-chromatography of IAA, IPA, 4Cl-IAA and IBA which had been separated on gradient 1 and then methylated).

Gradient 3: 10–45% B in A over 10 min, followed by isocratic elution at 45% B in A (used for neutral fractions containing typtophol and 3-indoleacetonitrile.

Gradient 4: 10–100% B in A over 30 min, followed by isocratic elution at 100% B (used for neutral fractions containing 4Cl-IAA methyl ester).

Gradient 5: 10–45% B in A over 10 min, isocratic elution at 45% B for 25 min then 45–100 B over 20 min (used for methylated unpurified acid ether fractions). The retention times of the acidic and neutral compounds on these gradients are listed in Table 1.

Thin-layer chromatography. The basic fraction from root tissue was examined for the presence of tryptamine using one-dimensional chromatography on Silica-gel plates (Whatman, Clifton, N.J., USA; K6). The plates were first washed in methanol to remove the fluorescent indicator. After heating at 100° C for 10 min to reactivate the plates, the samples were applied and the plates were run in *n*-butanol:ethanol:0.088 ammonia, 20:1:1 (by vol.) (Mulvena and Slaytor 1982). After drying for 5 min at 45° C, the plates were sprayed with Van Urk-Salkowski reagent (Ehmann 1977).

Derivatisation. Methylation was performed according to the method of Schlenk and Gellerman (1960). Nitrogen gas was bubbled through ether contained in a large stoppered test-tube (250 mm long, 20 mm diameter) and then into a second tube containing 7 ml diethyleneglycol monoethyl ether (carbitol),

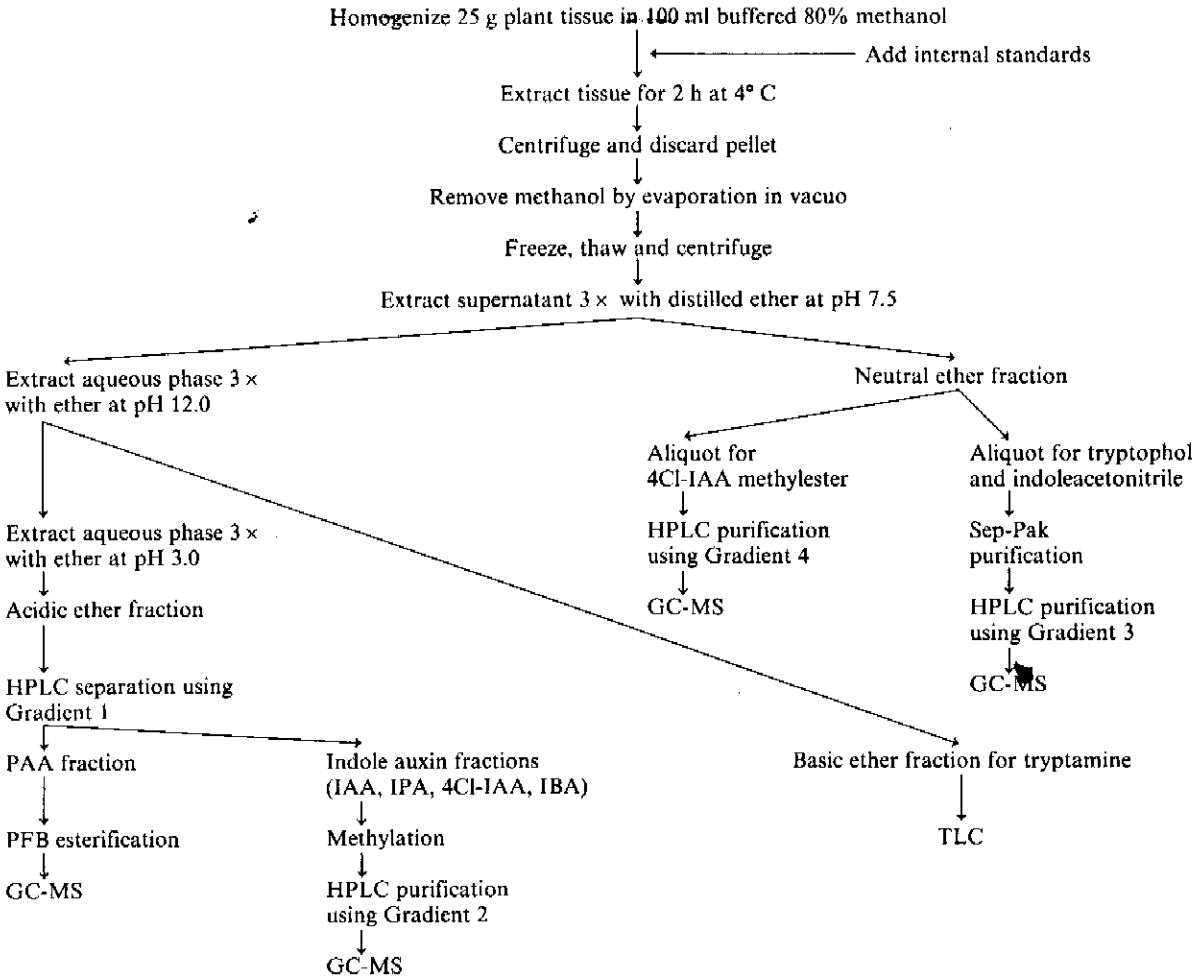

Fig. 1. Procedure for isolating and identifying auxins, auxin precursors and metabolites from pea seedling tissues

7 ml ether and 10 ml 10.5 M potassium hydroxide. N-Methyl-N-nitroso-*p*-toluene sulfonamide (1 g) was added to the second tube, generating diazomethane which was swept by the nitrogen stream into 20 ml of a 10% solution of methanol in ether until the solution was dark yellow. Two ml of the diazomethane solution were added to each sample contained in a glass vial (45 mm long, 15 mm diameter). The vials were capped for 30 min to ensure completion of the reaction and the solvent was then evaporated under nitrogen at 35° C, or allowed to evaporate overnight at room temperature in a fume hood.

Pentafluorobenzyl (PFB) esterification was performed according to Markham et al. (1980). Ten mg of anhydrous potassium carbonate and 0.5 ml of a solution of pentafluorobenzylbromide (PFBBr) in acetone (5 $\mu l\ ml^{-1}$) were added to each vial. The vials were capped with septum caps and heated in a Pierce Reactitherm heating module (Pierce Chemical Co., Rockford, Ill, USA) for 60 min at 60° C. The solvent was evaporated under nitrogen at 35° C and the residue was dissolved in 1 ml distilled water. The PFB esters were extracted from the aqueous solution with three 1-ml aliquots of ethyl acetate. The ethyl acetate was evaporated to dryness under nitrogen at 35° C.

Silylation was carried out by adding 0.5 ml of dry pyridine and 0.5 ml of bis-(trimethylsilyl)trifluoroacetamide (BFSTA) to each sample. The samples were then capped with septum caps and heated at 60° C for 60 min.

For a study of the stability of derivatised samples, the vials were capped wih septum caps and wrapped in Parafilm (CANLAB) to minimise evaporation. Methyl and PFB derivatives were stored in a freezer at −15° C and silylated samples were kept in a dessiccator over phosphorus pentoxide at room temperature in the dark.

Gas-liquid chromatography. Gas chromatography was performed using a Pye Series 104 gas chromatograph (CANLAB) equipped with dual-heated flame-ionisation detectors. Glass columns (2 m long, 2 mm internal diameter) were packed with 3% OV 101 on Chromosorb W HP, 80–100 mesh (Chromatographic Specialities, Brockville, Ont., Canada). Nitrogen was used as the carrier gas at a flow rate of 15 $ml\ min^{-1}$. The areas under the peaks were obtained using a Hewlett-Packard 3385A integrator (Hewlett-Packard (Canada), Ottawa, Ont.).

Gas chromatography–mass spectroscopy (GC-MS). Mass spectra were obtained using a VG 7070E instrument (VG Analytical, Manchester, UK). The glass column (2 m long, 2 mm internal diameter) was packed with 6% OV-17 on Chromsorb W HP. When full spectra were desired, the starting temperature

was 180 or 200° C and after a 2-min isothermal period, a temperature gradient of 10° C min^{-1} was applied.

The amounts of PAA, IAA and IPA present in the pea seedling were measured by single-ion monitoring (GC-MS-SIM). After homogenisation of the tissue, 10 μg of each deuterated standard (phenyl-$^2H_5$-acetic acid, indole-2,4,5,6,7-$^2H_5$-3-acetic acid or indole-2,4,5,6,7-$^2H_5$-3-propionic acid) were added to the homogenate. After purification by HPLC and derivatisation (Fig. 1), the PAA-PFB, IAA- and IPA-methyl esters were analysed by GC-MS-SIM, using the same OV-17 column packing as for full spectra. The PAA-PFB ester was run isothermally at 220° C and had a retention time of 3 min 15 s; the IAA- and IPA-methyl esters were run isothermally at 280° C and had retention times of 2 min and 2 min 20 s, respectively. The amounts of the endogenous compounds present could then be calculated from the $^2H_0/^2H_5$ ratio derived from the M^+-ion peak. No hydrogen exchange was found when the deuterated compounds were passed through the extraction and purification procedure. Both $^2H_4$- and $^2H_5$-IAA have shown to be preferable to $^2H_2$-IAA (labelled on the methylene carbon of the side-chain) as the side-chain deuterium is subject to loss (Magnus et al. 1980; Caruso and Zeisler 1983).

Bioassay of auxin activity. The growth-promoting activity of synthetic samples of the natural auxins studied in this investigation was determined in two tests which assay for the promotive activity on cell division or cell elongation.

1. *Pea lateral-root induction test:* After excision of a 5-mm apical segment, decapitated primary roots of 3-d-old pea seedlings. (*Pisum sativum* L., cv. Alaska) were treated with auxin solutions contained in glass vials, over the concentration range 10^{-4}–10^{-7} M. The vials and decapitated seedlings were supported in aluminium racks placed inside circular glass dishes and covered by a Petri dish top, as illustrated in Wightman et al. (1980). The seedlings in the dishes were incubated in the dark at 25° C for 3 d when the primary roots from each treatment of 20 seedlings were excised and placed in Petri dishes (140 mm diameter) containing 15 ml of 5% chromic acid. After 48 h, the cleared roots were placed over a light box and the average number of lateral roots in each sample was determined. The promotive activity of each auxin solution is expressed as the percent increase in number of lateral roots formed over that observed in water controls.

2. *Pea epicotyl segment test:* Epicotyl segments, 5 mm long, excised from the second internode of 6-d-old pea seedlings (cv. Alaska) were treated with auxin solutions over the concentration range 10^{-4}–10^{-7} mM potassium-phosphate buffer, pH 7.0. The seedlings were grown in vermiculite at 25° C under light from a 75-W red incandescent lamp located 60 cm above the plants, and the second internode was approx. 2 cm in length when the test segment was removed. Batches of ten segments were placed in Petri dishes on filter-paper bridges supported by two glass rods, the ends of the filter paper dipping into the test solution and serving as a wick for supplying solution to the segments. Segments were allowed to elongate in the dark at 25° C for 24 h when the average length of the ten segments in each solution was determined. Results are expressed as a percentage of the final length of buffer control segments, which showed an average length of 6.4 mm.

Sources of chemicals. Phenylacetic acid, IAA, IPA, 3-indolecacetonitrile, tryptophol, tryptamine, 3-indolepyruvic acid and N-methyl-N-nitroso-*p*-toluene sulfonamide were purchased from Sigma Chemical Co., St. Louis, Mo., USA; IBA was obtained from Regis Chemical Co., Chicago, Ill., USA; PFBBr and BFSTA were purchased from Pierce Chemical Co., Rockford, Ill., USA. 4Cl-IAA-methyl ester was a kind gift from Dr. K.C. Engvild (Riso National Laboratory, Roskilde, Denmark) and 4Cl-IAA was prepared from the methyl ester by Lee Collier, Chemistry Department, Carleton University. Phenyl-$^2H_5$-acetic acid, indole-2,4,5,6,7-$^2H_5$-3-acetic acid and indole-2,4,5,6,7-$^2H_5$-3-propionic acid were purchased from Merck, Sharp and Dohme, Isotopes Division, Pointe Claire, P.Q., Canada. All methanol used in extraction and chromatography was Baker Analysed HPLC grade (CANLAB) and all the ether used in extractions was freshly distilled reagent-grade diethyl ether.

Results and discussion

Methodology

Derivatisation. The principal methods which have been used for the derivatisation of auxins in preparation for gas-liquid chromatography are methylation (Powell 1964), trimethylsilylation (Davis et al. 1968; Liu et al. 1978) and methods using halogenated derivatives, such as pentafluorobenzylation (Markham et al. 1980; Epstein and Cohen 1981; Fregeau and Wightman 1983). Before selecting the most suitable derivatives for use in the present study, we investigated the stability of each of these derivatives during storage.

The stabilities of the PAA, IAA and IPA derivatives during three to four weeks of storage are shown in Table 2. The methyl derivative of PAA was not included since this compound is very volatile and is lost in varying amounts during evaporation of the reaction mixture to dryness. All the derivatives investigated were found to be essentially stable. However, it was necessary to maintain the silyl derivatives under rigorously dry conditions while the methyl and PFB derivatives do not suffer from this limitation.

McDougall and Hillman (1980) have shown that the yields of formation of the bis-trimethylsilyl and methyl derivatives of IAA were 100%. Epstein and Cohen (1981) also found complete conversion of IAA to the PFB ester in a reaction using α-bromopentafluorotoluene with N-ethyl-piperidine as a catalyst. We have found that the yield of the PFB ester of IAA prepared by the PFBBr-potassium carbonate method, as judged by the area of the peak obtained on the gas-liquid chromatogram, is very similar to that of the corresponding ester prepared by the method of Epstein and Cohen (1981), indicating that the PFFBBr method also gives a quantitative yield.

Silylation is the easiest and fastest of the three types of derivatisation to perform; the samples are ready for chromatography in about 1 h. Methylation is also rapid, but requires use of the toxic and potentially explosive diazomethane (Blau and King 1978). Pentafluorobenzylation is a slower procedure, requiring a heating period, extraction

Table 2. Stability of the pentafluorobenzyl (PFB), methyl (Me) and silyl (Si) esters of three auxins. Values represent the area of the peak obtained by GLC as a percentage of the peak area obtained from a freshly prepared sample. Each sample injection was 1 µl containing 10–50 ng of the auxin ester, depending on the type of ester under test

Compound	Time after preparation								
	1 week			2 weeks			3 weeks		4 weeks
	PFB	Me	Si	PFB	Me	Si	Me	Si	PFB
Phenylacetic acid	96	–	104	106	–	78	–	91	110
3-Indoleacetic acid	111	100	106	107	95	102	102	119	100
3-Indolepropionic acid	106	100	94	98	95	101	105	108	97

into ethyl acetate, and evaporation of the extract. Also, the PFBBr is a potent lachrymator and must be handled in a fume hood.

Methylated derivatives of the indole auxins can readily be separated by HPLC (Table 1, gradient 2), giving the opportunity for a second purification on a different gradient. Epstein and Cohen (1981) have used HPLC of IAA-PFB to purify the endogenous IAA from olive leaves. However, this procedure cannot be used for PAA-PFB as this compound is volatile and is lost during evaporation of the HPLC solvent in vacuo. Silyl derivatives are not suitable for HPLC because of their instability under aqueous conditions.

As mentioned in *Material and methods*, no hydrogen exchange occurred when $^2H_5$-PAA, -IAA or -IPA were passed through the purification procedure outlined in Fig. 1, which included the methylation of IAA and IPA and PFB esterification of PAA. Pentafluorobenzyl esterification of $^2H_5$-IAA and $^2H_5$-IPA resulted in an apparent hydrogen exchange of 0.23% and 0.22%, respectively. In a typical experiment, where 10 µg of each deuterated standard were added to 25 g FW of plant material, hydrogen exchange would therefore cause an overestimation of endogenous IAA and IPA of about 1 ng g^{-1} FW. When the levels of these compounds are low (particularly in the case of IPA) this could cause a substantial error.

Thus, methylation was chosen for the indole auxins since this reaction is simple, fast and high-yielding, and provides stable derivatives which can be further purified by HPLC and are not subject to hydrogen exchange during purification. As mentioned previously, however, the methyl derivative of PAA could not be used because it is too volatile; PFB esterification was found to be most satisfactory for this compound.

Recovery. When GC-MS-SIM with an internal deuterated standard is used as the method of quantitation, losses become less important as they are compensated for the internal standard. However, if losses are excessive, compounds may fall below the limits of detection; for this reason, we have tried to choose methods of purification which maximise auxin recovery. The recoveries of 5 µg amounts of standard PAA, IAA and IPA when passed through the purification procedure (Fig. 1) and measured by GC-MS-SIM are presented in Table 3.

Table 3. Percentage recovery of three auxins after purification through the isolation procedure outlined in Fig. 1

Auxin	Recovery (%)
Phenylacetic acid	58.2± 6.1
3-Indoleacetic acid	80.1± 9.0
3-Indolepropionic acid	24.3±12.8

The major problem in obtaining good recoveries of PAA is its volatility. In an attempt to reduce the number of steps requiring evaporation under vacuum, we tried to determine PAA in a crude acid ether extract, but found that the large number of compounds present made this impossible. As shown in Table 3, the recovery of PAA using one HPLC step was 58%. A second, isocratic HPLC step using 30% acetonitrile in 0.1 N formic acid reduced the recovery to 20% and did not result in an improved mass spectrum. Fortunately, most tissues contain relatively large amounts of PAA so that good spectra can be obtained after a single HPLC purification step.

Recovery of standard IAA in the absence of plant material was 80% (Table 3). Recovery was also measured in the presence of cotyledon tissue and was found to be 51%. For comparison, in some recent reports using similar methods, recovery of IAA was found to be 41% (Savidge et al. 1982, *Pinus* cambium), 15–30% (Dreher and Poovaiah 1982, strawberry fruits) and 30–40% (Allen et al. 1982, pea epicotyls).

Recovery of IPA was low and variable (Ta-

Table 4. Percentage recovery of different amounts of 3-indolepropionic acid after passage through the isolation procedure outlined in Fig. 1

IPA (μg)	Recovery (%)
1	2.8
5	18.6
10	33.2
25	57.2

ble 4). In addition, it was found that the percentage recovery of this compound was strongly dependent on the size of the sample. This behaviour indicates that some of the IPA may be adsorbed, probably at an early stage in the procedure when volumes are relatively large. Inspection of HPLC scans from the first chromatography showed that considerable loss had already occurred by that stage. As 25 g of plant material contain only about 0.025 μg of IPA (Table 5) recovery would be less than 2.8%. These low recoveries may explain why IPA has seldom been observed previously. The addition of the relatively large (10 μg) internal $^2H_5$-IPA standard should help to improve the recovery rate. Indolepropionic acid has sometimes been used as an 'internal standard' in the measurement of IAA (Akiyama et al. 1983); such studies assume that the behaviour of the two compounds during extraction is identical, something which we have not found to be true. In addition, the assumption is made that IPA is not an endogenous auxin while in fact it is widely distributed in plants (Table 5; Wightman 1977; Segal and Wightman 1982).

Auxin, auxin precursors and derivatives in pea seedlings

Mass spectra for phenylacetic acid were obtained from acidic ether extracts of all three organs of the seedling. The mass spectrum of the PFB ester of PAA isolated from epicotyl tissue is shown in Fig. 2; the mass ion at m/e 316, the $C_7H_2F_5$ fragment at m/e 181 and the base ion $-C_7H_7$ at m/e 91 can be seen. Phenylacetic acid has also been shown to be present in green pea leaves by bioassay and gas-liquid chromatography (Wightman and Lichty 1982), but has not previously been demonstrated in the root or cotyledon of any species. The PAA metabolite, N-phenylacetylaspartic acid, however, has been isolated from developing pea seeds (Gianfagna and Davies 1980). The level of PAA in all tissues was found to be relatively high (347–451 pmol g^{-1} FW; Table 5); on a molar basis, the PAA:IAA ratio in the whole plant was approx. 15:1, in the shoot 9:1, in the cotyledon 35:1 and in the root 3:1. The PAA:IAA ratio of green pea leaves as measured by gas-liquid chromatography was reported to be 4:1 (Wightman and Lichty 1982). Thus, although in auxin tests dependent on cell elongation, PAA is less active than IAA (see Table 8), because of its much greater concentration it may be of considerable physiological importance and in fact, about 40% of the total auxin activity in pea leaf extracts, as shown by the wheat coleoptile growth bioassay, was found at the R_f of PAA (Wightman and Lichty 1982). In addition, PAA is more active than IAA in the induction of lateral roots in pea seedlings (Wightman et al. 1980) (Table 8).

3-Indoleacetic acid was also found in all three organs of the pea seedling. The mass spectrum of the methyl ester of IAA isolated from root tissue showed the mass ion at m/e 189, the base (quinolinium) ion at m/e 130 and ions at m/e 103 and m/e 77 resulting from loss of -HCN and $-C_2H_2$ from the base ion (McDougall and Hillman 1980) (Table 6). Indoleacetic acid has previously been detected in etiolated pea epicotyls by gas-liquid chromatography (Bandurski and Schulze 1977) and by GC-MS-SIM (Allen et al. 1982). In the present study, the level of IAA was found to be highest in the root (115 pmol g^{-1} FW) and lowest in the cotyledons (13 pmol g^{-1} FW) (Table 5). The amount of IAA found in the epicotyl (46 pmol g^{-1}

Table 5. Concentrations of three endogenous auxins in the root, cotyledon and epicotyl of 3-d-old etiolated pea seedlings

Auxin	Root			Cotyledon			Epicotyl			Total
	ng g^{-1} FW	g^{-1} FW pmol	organ^{-1} pmol	ng g^{-1} FW	g^{-1} FW pmol	organ^{-1} pmol	ng g^{-1} FW	g^{-1} FW pmol	organ^{-1} pmol	(pmol plant^{-1})
Phenylacetic acid	47.2	347	23.8	61.4	451	180.1	58.1	427	31	234.9
3-Indoleacetic acid	20.1	115	7.9	2.3	13	5.1	8.0	46	2.3	15.3
3-Indolepropionic acid	0.9	5	0.3	0	0	0	0	0	0	0.3

Table 6. Comparison of the electron impact (70 eV) mass spectra of the methyl esters of suspected indole auxins isolated from pea roots with the methyl esters of the corresponding authentic auxins

Auxin	Fragmentation	Suspected methyl ester					Authentic methyl ester				
3-Indoleacetic acid	m/e	189	130	103	77	65	189	130	103	77	65
	Relative abundance (%)	30	100	12	12	6	30	100	8	12	7
3-Indolepropionic acid	m/e	203	142	130	114	88	203	142	130	114	88
	Relative abundance (%)	10	4	40	3	1.5	27	12	100	12	6
4-Chloro-3-indole-acetic acid	m/e	223/225	164/166	128	101	75	223/225	164/166	128	101	75
	Relative abundance (%)	30/10	100/36	15	16	10	30/9	100/37	13	12	5

FW) falls within the range reported by Allen et al. (1982) (30–90 pmol g^{-1} FW). As far as we are aware IAA levels in pea roots have not been measured previously. Ten-mm root tips of *Zea mays* contain 626 pmol g^{-1} FW (Rivier and Pilet 1974), which is several times higher than the values we found for peas. However, the values for whole roots might be considerably lower than those found in root tips. Although the concentration of IAA in the cotyledons is low on a fresh-weight basis, these organs contribute 70% of the fresh weight of the plant and 33% of total IAA.

A mass spectrum of the methyl ester of 3-indolepropionic acid isolated from pea roots showed the mass ion at m/e 203 and the quinolinium ion at m/e 130, as well as several minor contaminating ions (Table 6). Indolepropionic acid was reliably found only in the root. In the epicotyl, a weak mass spectrum for IPA was found in one out of four experiments, but it was never found in extracts from cotyledons. The level of IPA in the root was very low (5 pmol g^{-1} FW; Table 5).

4-Chloro-3-indoleacetic acid was found in all three organs. A mass spectrum of the methyl ester of 4Cl-IAA isolated from pea roots showed the mass ion at m/e 223/225 and the base ion at 164/166 (Table 6). These peaks have a 3:1 ratio of $^{35}Cl/^{37}Cl$ at m/e values two units apart, typical of chlorinated compounds (Pless et al. 1984). The endogenous level of this compound was not measured as we have not yet obtained a $^{2}H_5$-4Cl-IAA standard. Inspection of the HPLC scans, however, indicates that pea roots may contain up to 30 pmol g^{-1} FW, uncorrected for recovery. In this connection, Pless et al. (1984) have found that the recovery rate of 4Cl-IAA is similar to that of IAA, which in the present investigation was found to be approx. 80% (Table 3). 4-Chloro-*3*-indoleacetic acid was first isolated from immature pea seeds by Marumo et al (1968) and has recently been shown to be present in seeds and leaves of *Vicia faba* (Pless et al. 1984). In *Vicia* leaves, 4Cl-IAA levels were very high (77 nmol g^{-1} FW) but no IAA could be detected while, as shown in Table 7, both IAA and 4Cl-IAA are present throughout the pea seedling.

The naturally occurring methyl ester of 4Cl-IAA was found on one occasion in the neutral fraction of the pea root. It was absent in a second experiment and was not found in neutral fractions of the epicotyl or cotyledons. However, using a sample of frozen immature peas from a local supermarket, we confirmed the observation of Engvild et al. (1978) that large amounts of 4Cl-IAAMe are present in this tissue (data not shown). No naturally occurring methyl esters of IAA or IPA could be detected in the neutral fraction of the cotyledons, showing that these esters are not endogenous, nor are they formed during the methanol extraction process.

3-Indolebutyric acid was found in the root and epicotyl but not in the cotyledons. A mass spectrum of the methyl ester of IBA isolated from pea roots is illustrated in Fig. 3. As far as we are aware, this is the first unequivocal demonstration of endogenous IBA in the growing organs of a seedling plant, although suggestions of its occurrence in plants have appeared earlier (Blommaert 1954; Bayer 1969) and most recently, Badenoch-Jones et al. (1984) have identified IBA in pea root nodules by GC-MS. Since Sandberg et al. (1984) have demonstrated the presence of 3-indolecarboxylic acid (ICA) in *Pinus* needles by GC-MS, there is now evidence for the presence of the homologous series ICA, IAA, IPA and IBA in plant tissues. No quantitative estimations of IBA were made in the present work, but the appearance of the HPLC scans indicate that the level of this compound in pea tissues is low.

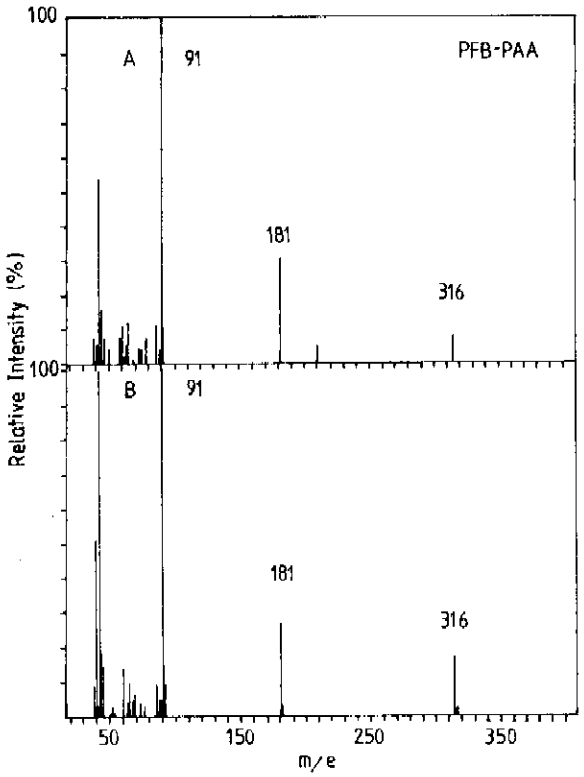

Fig. 2A, B. Mass spectra of the PFB esters of PAA isolated from pea epicotyls (**A**) and of authentic PAA (**B**)

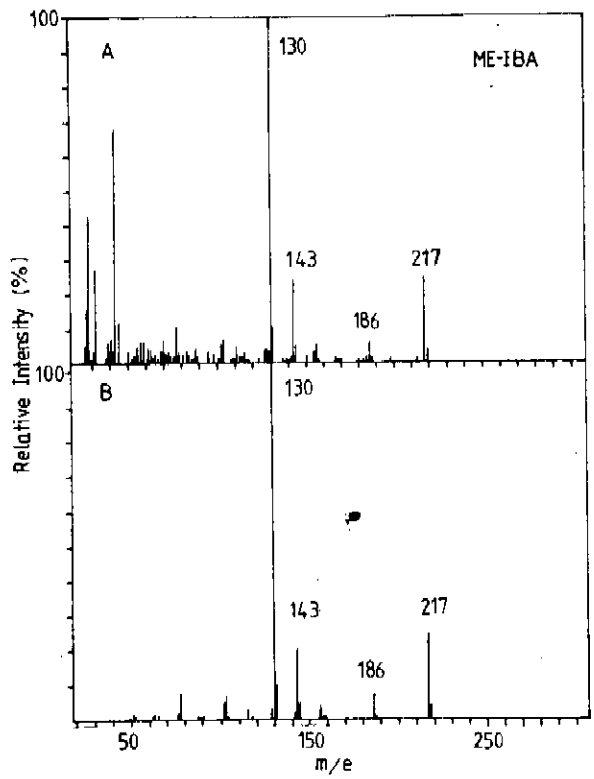

Fig. 3A, B. Mass spectra of the methyl esters of IBA isolated from pea roots (**A**) and of authentic IBA (**B**)

Table 7. Summary of the distribution of auxins, auxin precursors and metabolites in the root, cotyledon and epicotyl of 3-d-old etiolated pea seedlings

+ = present; mass spectrum obtained
(+) = present on one occasion
na = not analysed
– = absent

Compound	Seedling organ		
	Root	Cotyledon	Shoot
Phenylacetic acid	+	+	+
3-Indoleacetic acid	+	+	+
3-Indoleacetic acid methyl ester	–	na	na
3-Indolepropionic acid	+	–	(+)
3-Indolepropionic acid methyl ester	–	na	na
4-Chloro-3-indoleacetic acid	+	+	+
4-Chloro-3-indoleacetic acid methyl ester	(+)	–	–
3-Indolebutyric acid	+	–	+
3-Indolepyruvic acid	–	na	na
Tryptophol	–	na	na
3-Indoleacetonitrile	–	na	na
Tryptamine	–	na	na

Only the root tissue was analysed for the presence of the IAA precursors 3-indolepyruvic acid, tryptophol, 3-indoleacetonitrile and tryptamine. None of these compounds were found to be present. It should be noted, however, that the colorimetric procedure used for tryptamine had a detection limit of about 80 ng g^{-1} FW, which is far higher than that of GC-MS, so it is possible that low amounts of this compound may be present in the roots. As described in *Material and methods*, the sample containing 3-indolepyruvic acid was methylated before purification. It was found that methylation gave at least three compounds; GC-MS of the three peaks showed that two of the compounds were methyl-3-indolepyruvate and dimethyl-3-indolepyruvate. None of these compounds could be detected in the spiked plant sample after purification; thus, it is not clear whether 3-indolepyruvic acid is absent from the unspiked sample or whether it is lost during purification.

Conclusions

We have shown that pea roots contain five auxins, namely PAA, IAA, 4Cl-IAA, IPA and IBA (Table 7). All of these auxins are active in promoting the induction of lateral root primordia in primary roots of pea seedlings and in promoting cell elongation in pea epicotyl segments when applied exogenously (Table 8). Quantitative measurements of PAA, IAA and IPA have shown that IAA is present in amounts within the range previously found

Table 8. Growth-promoting activities of endogenous auxins when tested in bioassays dependent on the stimulation of cell division or cell elongation

Auxin tested	Pea lateral-root induction test[a]				Pea epicotyl segment test[b]			
	10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M	10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M
3-Indoleacetic acid	0	0	10*	46*	8*	27*	30*	32*
3-Indolepropionic acid	0	7*	13*	95*	2	8*	26*	32*
3-Indolebutyric acid	0	8*	15	85*	10*	24*	32*	35*
4-Chloro-IAA	10*	22*	44*	194*	26*	34*	36*	30*
3-Indoleacetonitrile	0	0	12*	35*	0	2	10*	14*
Phenylacetic acid	0	8*	18*	68*	0	0	6	16*

* Activity statistically significant from water control at the 0.1% level

[a] Activity is expressed as percent increase in number of lateral root primordia over the water control

[b] Activity is expressed as percent increase in length of epicotyl segment over buffer controls (increase 1.4 mm)

for this compound in plant tissues, while PAA is present in amounts 300% higher than those of IAA. The IPA levels, on the other hand, are only 4% of those of IAA.

Wightman and Thimann (1980) have shown that excision of the epicotyl or cotyledons appreciably affects the induction and emergence of lateral roots in the primary root of pea seedlings. This finding indicates that hormones may travel from these organs to the young root. We have found that the epicotyl contains PAA, IAA, 4Cl-IAA and IBA, while PAA, IAA and 4Cl-IAA are present in the cotyledons. Both these tissues are therefore possible sources of auxin hormones for the developing root. Martin et al. (1978) have shown that radioactivity from [^{14}C]IAA applied to the shoot of the *Zea mays* seedling is transported into the primary root, where it accumulates at the tip. We do not, however, know anything about the transport characteristics of the other endogenous auxins. We also need to know about the role of the root in synthesising its own auxins. Feldman (1980) has shown that sterile, isolated roots of *Z. mays* convert [^{14}C]tryptophan to [^{14}C]IAA, so it is possible that roots may also synthesise the other endogenous auxins.

The presence of IBA in pea root and epicotyl tissues is particularly interesting since we know of no previous reports of the isolation of this compound from developing plant organs, although the synthetic compound is well known as a promoter of both lateral and adventitious root formation. The question immediately arises as to the pathway of IBA biosynthesis since IBA has a 4-carbon side chain, while tryptophan, the precursor of most plant indoles has only a 3-carbon side chain. The pathway of biosynthesis of IBA is not immediately apparent. Similar questions may also be asked with respect to IPA; we have already found this compound to be present in squash hypocotyls (Segal and Wightman 1982), but its biosynthetic pathway is still unknown. Answers to these biosynthetic and transport problems should come from in vivo experiments using radioactive auxins and auxin precursors; these are currently underway in our laboratory.

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Communication

Measurement of Indolebutyric Acid in Plant Tissues by Isotope Dilution Gas Chromatography-Mass Spectrometry Analysis[1]

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ABSTRACT

An internal standard, [^{13}C][indole-2]-indole-3-butyric acid, was synthesized from indole-2[^{13}C] and was shown to be effective for the quantitative determination of indole-3-butyric acid from plant tissue. When this standard was used along with [$^{13}C_6$]indole-3-acetic acid, both indolic auxins could be quantified from the same tobacco (*Nicotiana tabacam*) leaf sample by isotope dilution analysis using selected ion monitoring gas chromatography-mass spectrometry for detection.

Auxins are important hormonal factors affecting adventitious root formation. In propagation systems, auxins, particularly in the form of IBA,[2] have been used to improve the percentage of rooting and quality of roots formed in both herbaceous and woody plants (3). IAA has been considered the primary endogenous auxin closely involved in adventitious root formation and has been found to be positively correlated with adventitious root formation (14). IBA, until recently thought of only in terms of its use as a synthetic growth regulator, has been found to be considerably more effective as an exogenous agent than IAA (1). Several hypotheses have been proposed to explain the greater effectiveness of IBA over that of IAA including (a) that IBA may be less susceptible to degradative enzymes than is IAA and (b) that over time IBA may be slowly converted to IAA, thus providing a steady supply of free IAA (2, 9). More recent studies by Nordstrom *et al.* (12) indicated that the greater effectiveness of IBA was due, in part, to its greater longevity within tissues.

Concepts about the relationship between IAA and IBA have changed with the recent demonstration that IBA is a native auxin occurring in maize, tobacco (*Nicotiana tabacam*) tumors, and carrot (4, 8, 10). To extend these findings, it has become important to devise accurate procedures to determine how much endogenous IBA contributes toward adventitious root formation as well as other physiological processes thought to be mediated by endogenous auxins. Thus, we set about the establishment of a method that would enable us to study the physiological involvement of both auxins by accurate quantitation of the levels of both IAA and IBA in plant tissues. In this report, we describe the production of stable isotope-labeled IBA and its application, together with [$^{13}C_6$] IAA, to the analysis of plant-derived IAA and IBA using stable isotope dilution GC-SIM-MS.

MATERIALS AND METHODS

Plant Material

Plants of tobacco (*Nicotiana tabacum* cv SRI) were grown from seed. When the plants were 4 weeks old, shoot tips were removed, surface sterilized using 0.05% sodium hypochlorite, and then placed in Murashige and Skoog nutrient medium containing 0.1 mg/L BA. Shoots were multiplied *in vitro* and were transferred every 4 weeks.

Synthesis of [^{13}C]Indole-[ring 2]-3-Butyric Acid

We used a modification of the method of Cohen and Schulze (7), described for the synthesis of ^{14}C-labeled IBA, for production of [^{13}C]IBA. Twelve milligrams of indole-2[^{13}C] (Cambridge Isotope Laboratories,[3] CLM-1863) were placed in a 1-mL (4 mL capacity) freeze-drying tear bulb (A.H. Thomas, 5136–610) fitted with a condenser collar. Freshly broken NaOH pellets (0.75 g) and 1.5 mL of γ-butyrolactone (Sigma) were added to the bulb. The tear bulb was placed in a heating mantle, brought to a temperature of 220°C at the rate of 2°C/min, and then refluxed for an additional 23-h period. The reaction was stopped by the addition of water and the reaction mixture was cooled to a hardened, white, solid glass. The product was then dissolved slowly by adding

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[2] Abbreviations: IBA, indole-3-butyric acid; SIM, selected ion monitoring.

[3] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply approval to the exclusion of other products or vendors that may be suitable.

2- to 5-mL aliquots of distilled water and shaking the mixture on a Vortex mixer each time. The final volume was 50 mL.

After the reaction mixture was dissolved, it was partitioned twice against an equal volume of chloroform. The aqueous phase was then adjusted to pH 2.5 with 6 N HCl and partitioned three times against diethyl ether. The ether extracts were combined and checked by TLC (7) for complete extraction of IBA into the ether phase. The chloroform and aqueous fractions were also checked for product. The chloroform fraction was found to contain substantial amounts of product, which was then partitioned against water and subsequently reextracted with diethyl ether. The ether fractions were combined and then dried over anhydrous sodium sulfate for 1 h, filtered, and evaporated to near dryness in a Buchi rotovaporator. The residue was applied to a 2.5 × 48 cm column of Sephadex LH-20 after being dissolved in 2 mL of 50% 2-propanol (aqueous, v/v). The IBA was eluted with 50% 2-propanol and collected in 16-mL fractions. IBA eluted at 276 to 294 mL as determined by TLC. These fractions were combined, dried *in vacuo*, and 2 mL of 2-propanol was added. The product was run on silica gel TLC (chloroform:methanol:water, 85:14:1 [v/v]; EM Science No. 5719–2 plates) against standards containing known amounts of IBA to determine approximate concentration and yield (30%, based on indole). Final concentration was determined by reverse isotope dilution analysis using a mixture of a known volume of the product solution and a known amount of unlabeled IBA, followed by methylation and GC-SIM-MS analysis (see below).

Determination of IBA and IAA Levels in Tobacco Tissues

Between 0.7 and 1.0 g fresh weight of leaf tissue was obtained from tobacco shoots grown *in vitro*. The tissue was ground in a liquid nitrogen-chilled mortar to a fine powder and 4 mL of imidazole buffer (35% 0.2 M imidazole, 65% 2-propanol, pH 7) per g of material was added. The ground plant material and buffer were transferred to a 13-mL Corex centrifuge tube and the following standards were added: 100,000 dpm [^{3}H]IBA (7 Ci/mmol, a gift from Dr. Ephraim Epstein, Volcani Center, Israel), 100,000 dpm [^{3}H]IAA (29 Ci/mmol, Amersham), and 50 ng each of [$^{13}C_6$]IAA (synthesized as in ref. 6) and [^{13}C][ring 2]-IBA. The extract was allowed to equilibrate with the added isotopes for 1 h at 4°C. The sample was centrifuged for 5 min at 2000*g* and was washed two additional times with extraction buffer. Extracts were combined and reduced *in vacuo* to one-third of their original volume.

The extract was transferred quantitatively to a Mixxor vessel (Lida, Israel) or to a small separatory funnel and partitioned once against one-half volume hexane. The aqueous phase was brought to pH 2.5 to 3.0 and was partitioned two times against diethyl ether, saving the ether phase. The ether portions were combined and dried over one-third volume anhydrous sodium sulfate for 1 h at 4°C. The ether phase was evaporated to dryness *in vacuo* and the residue dissolved in 100 μL of 15% acetonitrile.

HPLC Analysis

Samples were further purified by reverse-phase HPLC using a system consisting of two Waters 6000A pumps with a model 680 controller and fitted with a C_{18} reverse-phase, 3-μm Microsorb column (Rainin, 4.6 mm i.d. × 100 mm) and an Upchurch guard column packed with Whatman Co:Pell ODS. The gradient used was a linear change of acetonitrile/1% acetic acid from 15 to 50% acetonitrile over 25 min at 1 mL/min. Under these conditions, the retention volume of

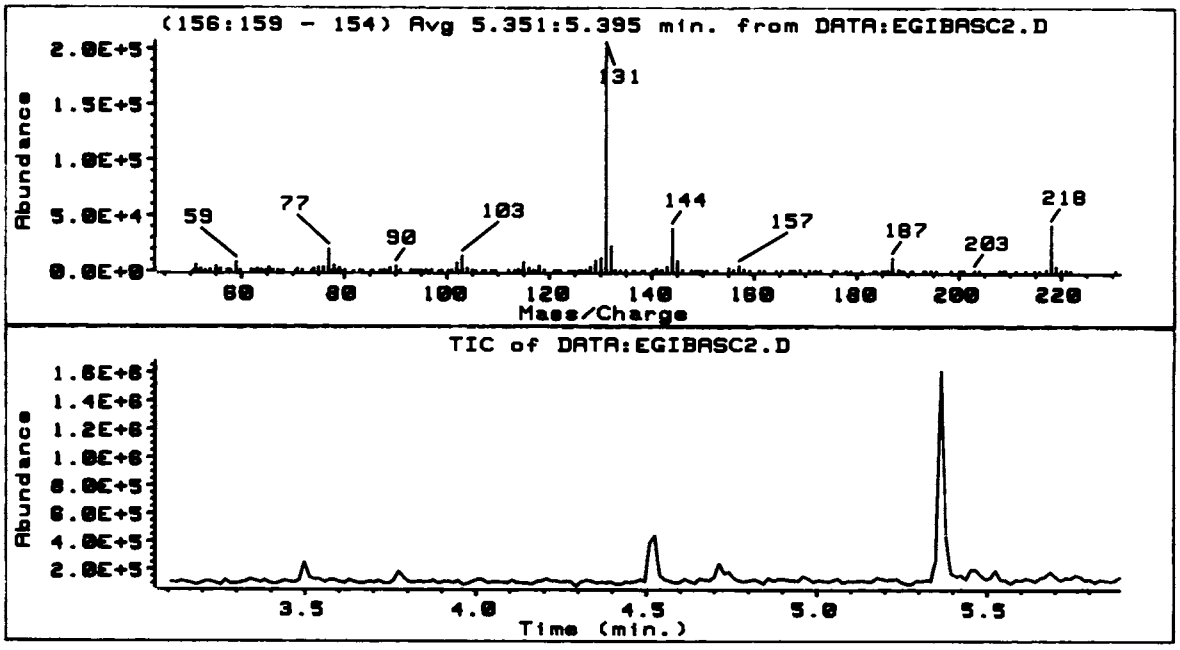

Figure 1. Mass spectrum and total ion chromatogram of synthesized [$^{13}C_1$]IBA methyl ester. The ions at m/z 131 and 218 are from the quinolinium ion and molecular ions, respectively.

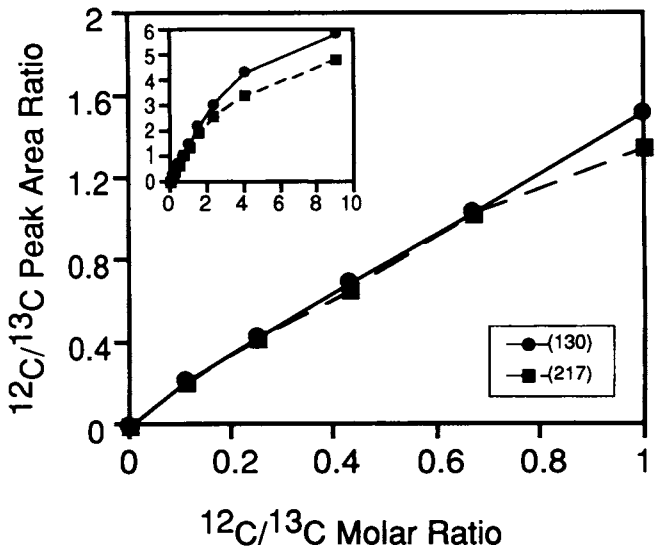

Figure 2. Calibration plot of [$^{13}C_1$]IBA showing the relationship between the molar ratio for methyl [$^{12}C/^{13}C$]IBA and the peak area ratios for methyl [$^{12}C/^{13}C$]IBA derived from the molecular and quinolinium ions.

IAA was 7.8 mL and that of IBA was 13.5 mL. Samples containing the radioactive peaks were combined, keeping the IAA and IBA samples separate. Each sample was evaporated to dryness *in vacuo* and 100 μL of methanol was added immediately. The samples were methylated with ethereal diazomethane (5), dried under nitrogen, and dissolved in 20 μL of ethyl acetate for analysis by GC-MS.

Analysis by GC-MS

GC-MS was performed using a Hewlett-Packard 5890 GC equipped with a 15 m × 0.21 mm i.d. DB-1701 fused silica column (J&W Scientific). Helium was used as the carrier gas at a flow rate of 1 mL/min. IAA and IBA were analyzed separately; however, the GC conditions were identical. The injector was at 250°C and the initial column temperature was 140°C. After a 1-min hold, the temperature was programmed to increase at a rate of 20°C/min. Under these conditions, methyl-IAA had a retention time of 4.7 min and methyl-IBA had a retention time of 6.5 min. On older columns, these times were reduced somewhat due to shortening of the column length (we routinely remove 0.33 m of column at the injector side periodically to maintain column performance) and a decrease in sample retention.

MS was performed with a Hewlett-Packard 5971A mass selective detector coupled to the GC using SIM with a dwell time of 50 ms for each ion. The selected ions monitored were at m/z 130, 136, 189, and 195 for IAA and its $^{13}C_6$ standard and m/z 130, 131, 217, and 218 for IBA and its $^{13}C_1$ standard.

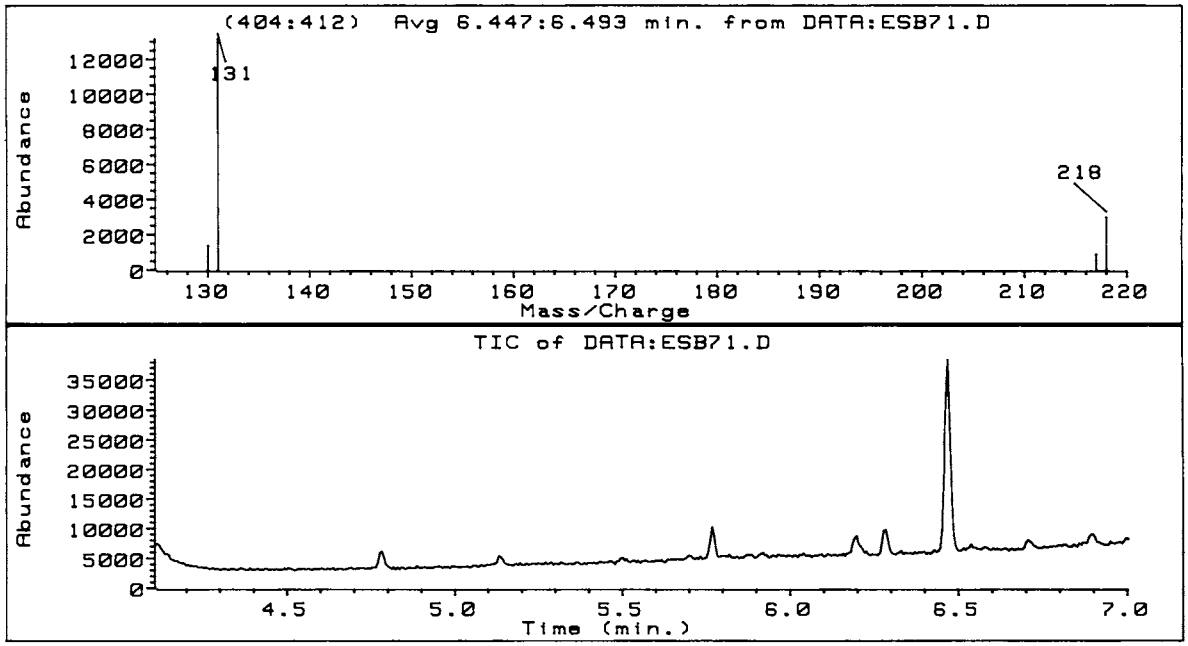

Figure 3. Mass spectrum and selected ion chromatogram of IBA fraction from partially purified and methylated extracts of tobacco leaf tissue using 100 ng of [$^{13}C_1$]IBA as internal standard.

RESULTS AND DISCUSSION

The identity of the synthesized $^{13}C_1$ standard was confirmed by GC-MS. The quinolinium ion at m/z 131 and the molecular ion at m/z 218 were present as expected with no significant ^{12}C isotopic contribution at m/z 130 and 217 (Fig. 1). In addition, an essentially linear relationship was observed between the molar ratio and area ratio of $^{12}C/^{13}C$ (Fig. 2). One potential problem with using a stable isotope-labeled standard with only one heavy atom is that it overlaps with the naturally occurring heavy isotope cluster on the plant-produced compound (11, 13). Thus, when the internal standard was significantly less than the sample, nonlinearity occurred (Fig. 2, inset). This was primarily due to the contribution of ^{13}C that occurs naturally in unlabeled IBA. The amount of ^{13}C in unlabeled IBA accounts for 10.28% of the ion at m/z 130 (13). However, because the internal standard we produced contains no significant amount of unlabeled IBA, near linearity is obtained as long as the ratio of internal standard to endogenous IBA in the sample is high. Under conditions in which the amount of internal standard added is high relative to what was in the plant sample, only a minor correction (*i.e.* subtraction of 10.28% of the m/z 130 value from the m/z 131 abundance) is required to account for the small overlap at m/z 131 and 218. In other situations in which a higher endogenous level of IBA is found than was expected, the quantitation can be calculated using the known relationship (see ref. 11) or, more conveniently, the standard curve can be used for quantitation.

The mass spectrum of the tobacco leaf sample showed a clear peak at the retention time for IBA (Fig. 3). The selected ion mass spectrum of the peak had m/z values at 130, 131, 217, and 218, confirming that the peak was IBA. For the sample shown, the values obtained for IAA were 26 ng/g fresh weight free and 52 ng/g fresh weight total; for IBA they were 9 ng/g fresh weight free and 37 ng/g fresh weight total.

The use of [$^{13}C_1$]IBA as an internal standard is similar to the previous use of [$^{13}C_6$]IAA (6) for measurement of IAA, except as noted above. Previously, no heavy-labeled internal standard was available of analysis of IBA. The availability of [$^{13}C_1$]IBA now allows the determination of the amount of IBA in plant tissues with the precision and reliability inherent in the stable isotope dilution GC-SIM-MS technique.

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