NOTE TO USERS

Title of Thesis: Analysis of indole-3-butyric acid auxin activity in Arabidopsis

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January 2004

A thesis submitted to the Faculty of Graduate studies and Research in partial fulfillment of the requirements of the degree of Philosophae Doctor in Biology

Your file Votre référence
ISBN: 0-612-98352-8
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ISBN: 0-612-98352-8

Abstract:

Auxins are plant hormones involved in virtually all aspects of plant life. Despite long-term commercial and horticultural use of the auxin Indole-3-Butyric Acid (IBA), a full recognition of its natural occurrence in plants was made only recently. I have used multiple approaches to dissect the role of IBA in *Arabidopsis thaliana*. This thesis includes the first characterization of a mutant with an altered response to IBA that retains wild-type sensitivity to Indole-3-Acetic Acid (IAA), the most studied endogenous auxin. This mutant, named *resistant to IBA* (*rib1*), has modified root architecture and gravitropism and is resistant to auxin transport inhibitors. As these phenotypes are reminiscent of those of characterized auxin transport mutants, movement of IAA and IBA was studied in wild-type and mutant plants. IBA is transported in seedlings in three distinct flows, like IAA, and this transport is saturable, indicating it is carrier mediated. However, unlike IAA, IBA is not polarly transported in inflorescence axes, and IBA transport is not sensitive to IAA transport inhibitors. These results suggest IAA and IBA transport could be mediated or regulated by different mechanisms. In *rib1* seedlings, all flows of IBA transport are modified, while IAA transport levels are unchanged. Modifications in IBA transport match phenotypic differences between *rib1* and wild-type, and analyses of the physiological effects of IBA also suggest IBA has a role in defining wild-type seedling morphology in Arabidopsis. Though IAA transport levels are not changed in *rib1*, one flow of IAA transport is rendered insensitive to IAA transport inhibitors, perhaps revealing cross-talk between IAA and IBA transport regulation. Additionally, double mutant analyses reveal that IAA transport and response mutants can suppress some phenotypes of *rib1*, and some mutant combinations produce novel phenotypes, further suggesting cross-talk between IBA and IAA transport and response pathways. Finally, I found that the light signaling *hy5* mutant shares many of the same phenotypes as *rib1* and double mutant analysis suggests HY5 could be partially redundant with RIB1 in regulating many of these. More complete analysis of double mutants could further define pathways in which RIB1, and IBA transport, play an important role.

Résumé

Les auxines sont des hormones végétales impliquées dans presque tous les aspects de la vie de la plante. Bien que l'acide indole-3-butyrique (AIB) soit utilisée depuis longtemps à des fins commerciales et horticoles, sa présence comme auxine endogène des plantes n'a été pleinement reconnue que récemment. J'ai utilisé plusieurs approches afin de définir le rôle de l'AIB chez *Arabidopsis thaliana*. Cette thèse contient la première caractérisation d'un mutant présentant des défauts de réponse à l'AIB, tout en conservant une réponse normale à l'acide indole-3-acétique (AIA), l'auxine endogène la plus étudiée. Ce mutant, nommé «*resistant to IBA1*» (abbrévié *rib1*), présente une architecture et un gravitropisme des racines modifiés et est résistant aux inhibiteurs de transport de l'AIA. Comme ces phénotypes rappellent ceux de mutants bien caractérisés de transport d'auxine, nous avons étudié le transport de l'AIA et de l'AIB chez des plantes mutantes *rib1* et de type sauvage. Tout comme l'AIA, l'AIB est transporté dans les plantules via trois flux distincts. Ce transport est saturable, indiquant qu'il est médié par des transporteurs. Cependant, contrairement à l'AIA, l'AIB n'est pas transporté de façon polaire dans les tiges florales, et le transport de l'AIB n'est pas inhibé par les inhibiteurs de transport de L'AIA. Ces résultats suggèrent que des mécanismes différents pourraient médier ou réguler le transport de l'AIA et de l'AIB. Chez les plantules *rib1*, tous les flux de transport de l'AIB étaient affectés, alors que les niveaux de transport de l'AIA n'étaient pas différents du type sauvage. Les différences de transport de l'AIB concordaient avec des différences phénotypiques entre les plantules mutantes et de type sauvage, et des analyses physiologiques des effets de l'AIB suggèrent également que l'AIB joue un rôle dans la définition de la morphologie normale des plantules. Même si les niveaux de transport de l'AIA n'étaient pas affectés par la mutation *rib1*, un flux de transport de l'AIA devenait insensible aux inhibiteurs de transport de l'AIA, révélant ainsi des interactions possibles entre la régulation du transport de l'AIA et celle de l'AIB. De plus, des analyses de double mutants ont révélé que des mutants de transport et de réponse à l'AIA pouvaient supprimer certains phenotypes de *rib1*, et certaines combinaisons de mutants produisaient des phénotypes nouveaux, suggérant aussi des liens entre les voies de réponse et de transport des deux auxines. Finalement, j'ai trouvé que le mutant de réponse à la lumière *hy5* partageait plusieurs phénotypes avec *rib1*, et l'analyse de double

mutants suggère que HY5 et RIB1 pourraient être partiellement redondants dans la régulation de ces phénotypes. Une analyse plus exhaustive des double mutants pourrait permettre de mieux définir les voies dans lesquelles RIB1, et le transport de l'AIB, jouent un rôle important.

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Abbreviations used

1-NOA	1-Naphthoxyacetic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
2,4-DB	2,4-Dichlorophenoxybutyric acid
ABA	Abscisic acid
ACC	1-Aminocyclopropane-1-carboxylic acid
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BA	Benzyladenine
CAPS	Cleaved amplified polymorphic sequences
CoA	Coenzyme A
Col-0	Wild-type Columbia ecotype of *Arabidopsis thaliana*
CSI	Chromosaponin I
Cys	Cysteine
d	day(s)
d.n.s.	Data not shown
d.a.g.	Days after germination
FAD	Flavin adenine dinucleotide
FADH	Reduced FAD
GC-MS	Gas-chromatography-Mass spectrometry
GUS	ß-Glucuronidase
h	Hour(s)
HFCA	9-Hydroxyfluorene-9-carboxylic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
Ler	Landsberg *erecta* ecotype of *Arabidopsis thaliana*
MeJA	Methyl jasmonate
min	Minute(s)
NAA	Naphthalene acetic acid

NAD	Nicotinamide adenine dinucleotide
NADH	Reduced NAD
No-0	Wild-type Nossen ecotype of *Arabidopsis thaliana*
NPA	1-Naphthylphthalamic acid
PAA	Phenylacetic acid
PTS	Peroxisomal targeting sequence
SD	Standard deviation
SE	Standard error
SSLP	Simple sequence length polymorphism
TIBA	2,3,5-Triiodobenzoic acid
Ws	Wild-type Wassilewskija ecotype of *Arabidopsis thaliana*
WT	Wild-type

Acknowledgements

I first and foremost want to thank my supervisor, Candace Waddell, for her support throughout the length of my studies in her laboratory, and during the writing of my thesis. She introduced me to the wonderful world of Arabidopsis genetics, and her confidence in me encouraged me to pursue this thesis to its conclusion. Her acute critical sense helped make me a better scientist, and a stronger person. Finally, her understanding helped me persevere and motivated me to continue.

I must also acknowledge the financial help Candace has given me: she helped me throughout the years, but I am especially grateful for the first weeks and last stretch of my thesis work, when no other funding was available to me. My first years at McGill were funded by doctoral scholarships from the Natural Sciences and Engineering Research Council of Canada (NSERC), and then from the Fonds pour la formation des chercheurs et l'aide à la recherche (FCAR). I also wish to acknowledge funding received from McGill, in the form of the Arthur Willey admission scholarship from the Biology department, and the J.W.McConnell McGill major scholarship. Without these important funds, this thesis could not have been pursued to completion.

I also want to warmly thank Paul Lasko, who accepted at the last minute to become my co-supervisor, despite his already overcharged schedule. Many thanks go to Susan Bocti, who throughout the years has taken good care of us graduate students, and has been especially helpful with all the paper work related to thesis submission. It probably would have taken a few more weeks without her!

I am grateful for having had great colleagues in Candace's laboratory: fellow graduate students Aaron and Andy, with whom we had a lot of fun, and also all the undergraduates, who livened up the lab. I particularly wish to thank Marianne Marcoux and Tanya Radforth, for excellent technical assistance in performing the experiments described in this thesis.

I wish to thank the members of the Image center, Carole and Guy for their professionalism, and Robert Lamarche for his help with all computer related problems.

I have also been lucky to work with Gloria K Muday and Aaron M Rashotte, who have been key players in our investigation of IBA transport, and are great collaborators. I also wish to thank members of my graduate training committee, professors Greg Brown

and Tom Bureau, who have helped to keep me on track at yearly meetings. A special thank you goes to professor Rajinder Dhindsa, who challenged me at my qualifying exam, and has also been very supportive throughout the years.

I want to thank members of the Brown, Dhindsa, Bureau and Sieburth lab for occasionally lending me equipment, journals, and for being great colleagues; they made my years at McGill even more enjoyable.

Andy, Lily and Hien are great friends I made at McGill, and I hope we stay in touch for the long run! Thank you for your support, but also for your technical and scientific advice. Merci à ma famille et à mes amis d'être présents pour moi.

Enfin, je tiens à remercier tout particulièrement Naguib, qui me soutient et m'encourage depuis notre première année d'université. T'M. Merci à Florence pour ses sourires et ses jokes.

Publications and presentations at scientific meetings based on the work presented in this thesis:

Refereed publications:

Chapter 2, "The *rib1* mutant is resistant to indole-3-butyric acid, an endogenous auxin in *Arabidopsis thaliana*", by Julie Poupart and Candace S. Waddell, has been published in 2000 in Plant Physiology vol. 124, pp. 1739-1751.

Chapter 3, "Transport of two natural auxins, indole-3-butyric acid and indole-3-acetic acid, in Arabidopsis", by Aaron M. Rashotte, Julie Poupart, Candace S. Waddell and Gloria K. Muday, has been published in 2003 in Plant Physiology vol. 133, pp. 1-12

Presentations at scientific meetings:

1998: Oral presentation: "Characterization of the *iba1* mutant of *Arabidopsis thaliana*, a mutant affected in auxin response, gravitropism and lateral root formation." Authors: Julie Poupart and Candace S. Waddell. Annual Meeting of the Canadian Society of Plant Physiology, held in Montreal Canada, July 13th 1998.

1999: Poster presentation: "Characterization of *rib1*, and IBA specific mutant of *Arabidopsis*." Authors: Julie Poupart and Candace S. Waddell. Annual Meeting of the American Society of Plant Physiology, held in Baltimore, MD, USA July 24-28, 1999.

2000: Poster presentation: "*rib1*, an Indole-butyric acid resistant mutant of Arabidopsis". Authors: Julie Poupart and Candace S. Waddell. 6th International Congress of Plant Molecular Biology held June 18-24 2000, in Québec city, Canada,

Contributions of the author

Chapters 1, 2, 5 and Appendix 1 are my original work. Two communications in this thesis are the result of collaborative work with the laboratory of Professor Gloria K Muday, Wake Forest University. This section details my contribution to these papers.

Chapter 3: IAA and IBA transport in wild-type plants

Rashotte, AM, Poupart, J, Waddell, CS, and Muday, GK (2003) Transport of the Two Natural Auxins, IBA and IAA, in Arabidopsis. Plant Physiology, 133: 1-12.

As second author of this article, I performed the auxin transport assays in inflorescence stems, performed the experiments and analyzed the results of physiological activity of IBA on hypocotyl elongation and contributed significantly to writing of the paper.

Chapter 4: Auxin transport in *rib1* plants

Poupart, J, Rashotte, AM, Muday, GK, and Waddell, CS, (2003) A specific defect in IBA transport results in modifications in hypocotyl elongation and root architecture in Arabidopsis. Manuscript in preparation

As first author of this article, I performed the auxin transport assays in inflorescence stems, performed the experiments and analyzed the results of physiological activity of IBA and am the main author of the manuscript.

Chapter 1: Introduction

Chapter 1: Introduction

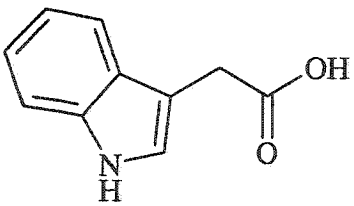
Auxins are a class of plant growth regulators that have ubiquitous roles in plant growth and development. They have been involved in such diverse functions as cell division and elongation, gravity response, lateral root formation and embryo patterning (Davies, 1995). In agriculture and agronomy, these growth regulators are used to control fruit production, to promote root formation on cuttings, or even as herbicides, among other applications (Gianfagna, 1995). Molecules are defined as auxins based on their physiological activity rather than by a given chemical structure. Many forms of auxin exist: examples of molecules that possess auxin activity can be seen in Figure 1.1. Indole-3-Acetic Acid (IAA) was the first auxin to be identified in plants, and comparatively little attention has been paid to the role of other endogenous auxins over the years. We have used physiological, genetic and molecular approaches to study the role and activities of one such endogenous auxin, indole-3-butyric acid (IBA), in the model system *Arabidopsis thaliana.*

IBA as an endogenous auxin

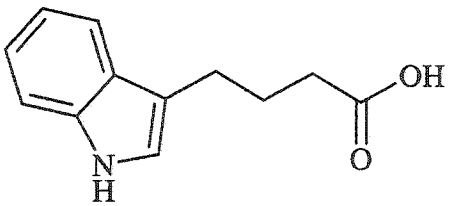
Zimmerman and Wilcoxon discovered the auxin activity of IBA as early as 1935, in a search for root promoting compounds with activities similar to those of IAA. Many chemically synthesized molecules were tested for activity, and IBA was found to be a more effective inducer of adventitious roots than IAA in a variety of plant species (Zimmerman and Wilcoxon, 1935). IBA is still the auxin of choice to induce root formation on cuttings (Blazich, 1988; Hartmann et al., 1997). Interestingly, even though IBA was also found as a natural constituent of plants by paper chromatography as early as 1954 (Blommaert, 1954), it retained the label of "synthetic auxin" in plant physiology textbooks until recently because it had first been "discovered" by chemical synthesis (see for example Taiz and Zeiger, 1998, which lists IBA as a synthetic auxin).

The occurrence of IBA at physiologically relevant concentrations in many plant species was confirmed by gas chromatography-mass spectrometry (GC-MS), and levels

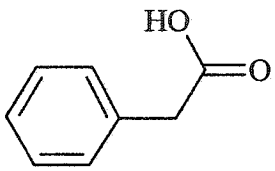
Endogenous auxins:



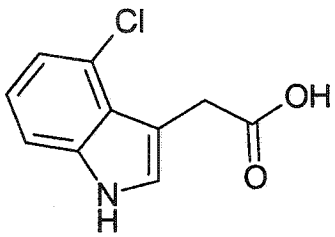
IAA
Indole-3-Acetic Acid



IBA
Indole-3-Butyric Acid

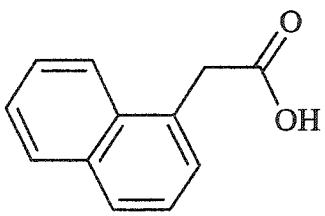


PAA
Phenylacetic Acid

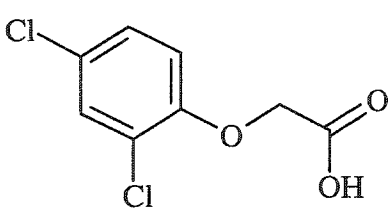


4-Cl-IAA
4-Chloroindole-3-Acetic Acid

Synthetic auxins:



NAA
Naphthalene Acetic Acid



2,4-D
2,4-Dichlorophenoxyacetic Acid

Figure 1.1: Chemical structure of selected natural and synthetic auxins.

of IBA are as high as IAA levels in some plant tissues (reviewed in Epstein and Ludwig-Müller, 1993; Ludwig-Müller, 2000).

Auxin levels in a single cell will depend on the amount of synthesis and catabolism, but also on the level of transport and conjugation. Conjugation is the process through which IAA and IBA are covalently bound to sugars or amino acids (Normanly, 1997; Sembdner et al., 1994). The resulting conjugated pools of auxin act as storage forms of auxin that can be released as needed by specific hydrolases. IBA can be produced from IAA under certain conditions (Ludwig-Müller and Epstein, 1994; Ludwig-Müller and Hilgenberg, 1995; Ludwig-Müller et al., 1995a; Ludwig-Müller et al., 1995b), and, like conjugates, IBA can also be converted to IAA in plants (Bartel et al., 2001). The auxin activity of IBA had therefore generally been assumed to depend on its conversion to IAA, in a manner similar to conjugates. It was shown early on that molecules similar to IAA and 2,4-D, but with longer aliphatic side chains containing an odd number of methylene (CH_2) groups (such as IBA and 2,4-DB) can be shortened by two carbons at a time to yield the auxin IAA or 2,4-D, while derivatives with even-numbered chains do not yield active auxin. This suggested a process such as ß-oxidation of fatty acids, which shortens the aliphatic chain of fatty acids and releases the two carbon molecule acetyl-CoA, could be responsible for shortening of the side chain of auxin precursor molecules (Fawcett et al., 1960; Wain and Wightman, 1954). The fact that IBA was considered as yet another slow release form of IAA, like conjugates, could explain in part why little attention has been paid to this endogenous auxin over the years. The possible interconversion of IAA and IBA renders elucidation of the specific role of IBA by physiological studies experimentally difficult.

When I first joined the Waddell laboratory, I began studying a mutant line that had been identified because it was defective in root gravitropism. We soon discovered that this mutant was resistant to IBA (and 2,4-D) but had a wild-type response to IAA. At that time, in 1997, there were no reports of Arabidopsis mutants specifically resistant to IBA; our results were unlike all other published reports of auxin resistant Arabidopsis mutants, which were all resistant to IAA, in addition to 2,4-D. In most cases, the response to IBA (still considered a "synthetic auxin") was not investigated. The publication of the paper presented in Chapter 2, titled "The *rib1* mutant is resistant to

indole-3-butyric acid, an endogenous auxin in *Arabidopsis thaliana"*, was the first report of the phenotypic characterization of a mutant specifically defective in IBA response. It was published the same month as another paper (in the journal Genetics) reporting the results of a screen aimed at isolating IBA resistant mutants, by Bonnie Bartel's laboratory. Since then, other IBA mutants have been characterized, and other previously identified classes of Arabidopsis mutants have been shown to have defects in IBA response, as detailed in the next section.

Survey of IBA resistant mutants

In order to better understand the role and mode of action of IBA in plants, we and others have studied Arabidopsis mutants[1] defective in response to IBA, but retaining wild-type sensitivity to IAA (Poupart and Waddell, 2000 - Chapter 2 of this thesis; Zolman et al., 2000). The existence of such mutants reveals that IAA and IBA sensitivity are genetically separable, and IBA resistant mutants can therefore be used to genetically dissect the role and functions of IBA from those of IAA. Most IBA resistant mutants will be described in the following text, and Table 1.1 also lists these mutants, their phenotypes and the genes affected by the mutations, when they have been identified. Mutants isolated in other screens that are also IBA resistant and IAA sensitive are also described here, and have been included in the list of Table 1.1. Some mutants, such as the *rib1* mutant described in this thesis, suggest IBA does not act solely through conversion to IAA. Many other IBA resistant mutants are affected in ß-oxidation enzymes, thus validating the theory that IBA also acts, at least in part, through conversion to IAA.

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[1] Throughout this study, we follow the convention in Arabidopsis community standards for mutant, gene and protein designation: mutant names are italicized and written in small letters, and alleles are designated by different numbers following a dash (e.g. *axr1-1* and *axr1-2* are two allelic mutations); gene names are always italicized, and the wild-type gene is designated by capital letters (e.g. the *AXR1* gene is affected by the *axr1-1* mutation); finally, protein names are written in non-italicized capital letters (e.g. AXR1 designates the wild-type protein) (Standards defined in Meinke and Koornneef, 1997).

ß-oxidation mutants

ß-oxidation is a spiral process by which carbon chains are shortened by two carbons through 4 successive steps (see Figure 1.2). The first (oxidative) step is catalyzed by a family of acyl-CoA oxidases in Arabidopsis. The six Arabidopsis acyl-CoA oxidases have different chain length specificities, though some overlap in specificity exists (Rylott et al., 2003, and references therein). The next two steps of ß-oxidation, a hydration and a second oxidation, are catalyzed by an enzyme called the Multifunctional protein (MFP). There are at least two members of the MFP family in the genome of Arabidopsis: *AIM1* and *MFP2*. These differ in expression pattern; *MFP2* is most strongly expressed in seedlings and senescing leaves, while *AIM1* is only weakly expressed in seedlings before day 8, and has stronger expression in mature tissues, notably siliques and flowers (Eastmond and Graham, 2000; Richmond and Bleecker, 1999). Finally, the last step of ß-oxidation is catalyzed by a thiolase that releases acetyl-CoA and produces an acyl-CoA that is shorter by two carbons (acyl-CoA #2 in Figure 1.2) than the initial acyl-CoA (#1). The ß-oxidation spiral can be repeated again with this shorter acyl-CoA, as long as it contains at least two carbons that can be removed by the next turn of ß-oxidation.

A model for how IBA is converted to IAA in peroxisomes is presented in Figure 1.3. In parallel, this figure shows how the inactive auxin precursor 2,4-dichlorophenoxybutyric acid (2,4-DB) is converted to the auxin 2,4-D by a very similar mechanism. 2,4-DB has been used extensively as a selective agent to isolate ß-oxidation mutants (see for example Hayashi et al., 1998; Lange and Graham, 2000).

In plants, most ß-oxidation occurs in peroxisomes, which are single membrane-bound specialized organelles in which many essential metabolic processes occur in most eukaryotes (see review in Johnson and Olsen, 2001). The physiological function of peroxisomes varies depending on the tissue, and on the metabolic or developmental stage of the organism. Young seedlings contain a specialized form of peroxisomes called glyoxysomes, which, in oilseeds such as Arabidopsis, are used to convert stored oil into energy for growth prior to the establishment of photosynthesis. Figure 1.4 presents a schematic view of the utilization of seed oil reserves in a plant cell prior to establishment of photoautotrophic growth in seedlings. Glyoxysomes contain, in addition to

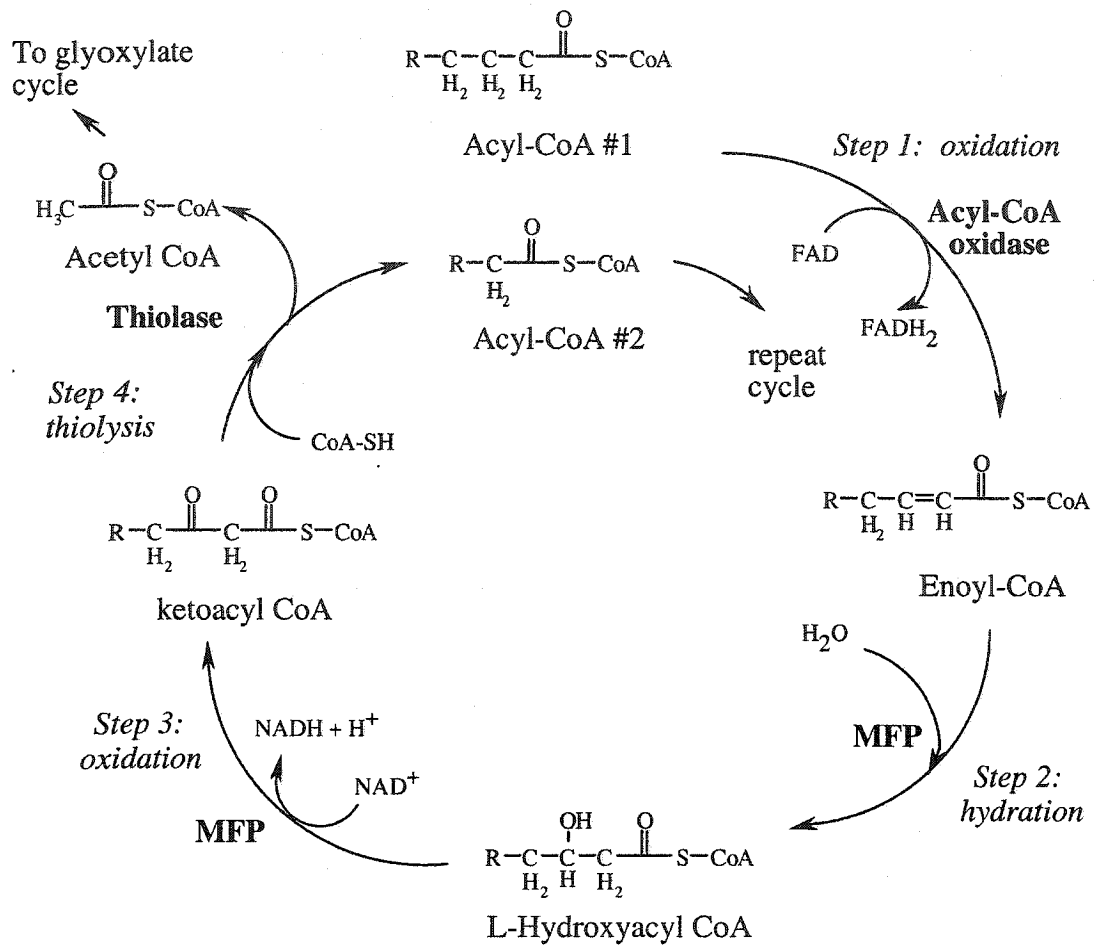

Figure 1.2: ß-oxidation spiral: ß-oxidation is a four step spiral process by which carbon chains are shortened by two carbons at a time. The first step, an oxidation, is catalyzed by a family of acyl-CoA oxidases. Steps 2 and 3 are catalyzed by an enzyme called the Multifunctional Protein (MFP). The last step is catalyzed by a thiolase. The products of thiolysis are an Acetyl-CoA and an Acyl-CoA with a 2 C shorter side chain. The spiral can be repeated until a single or no methylene group remains on Acyl-CoA #2. See text for more details.

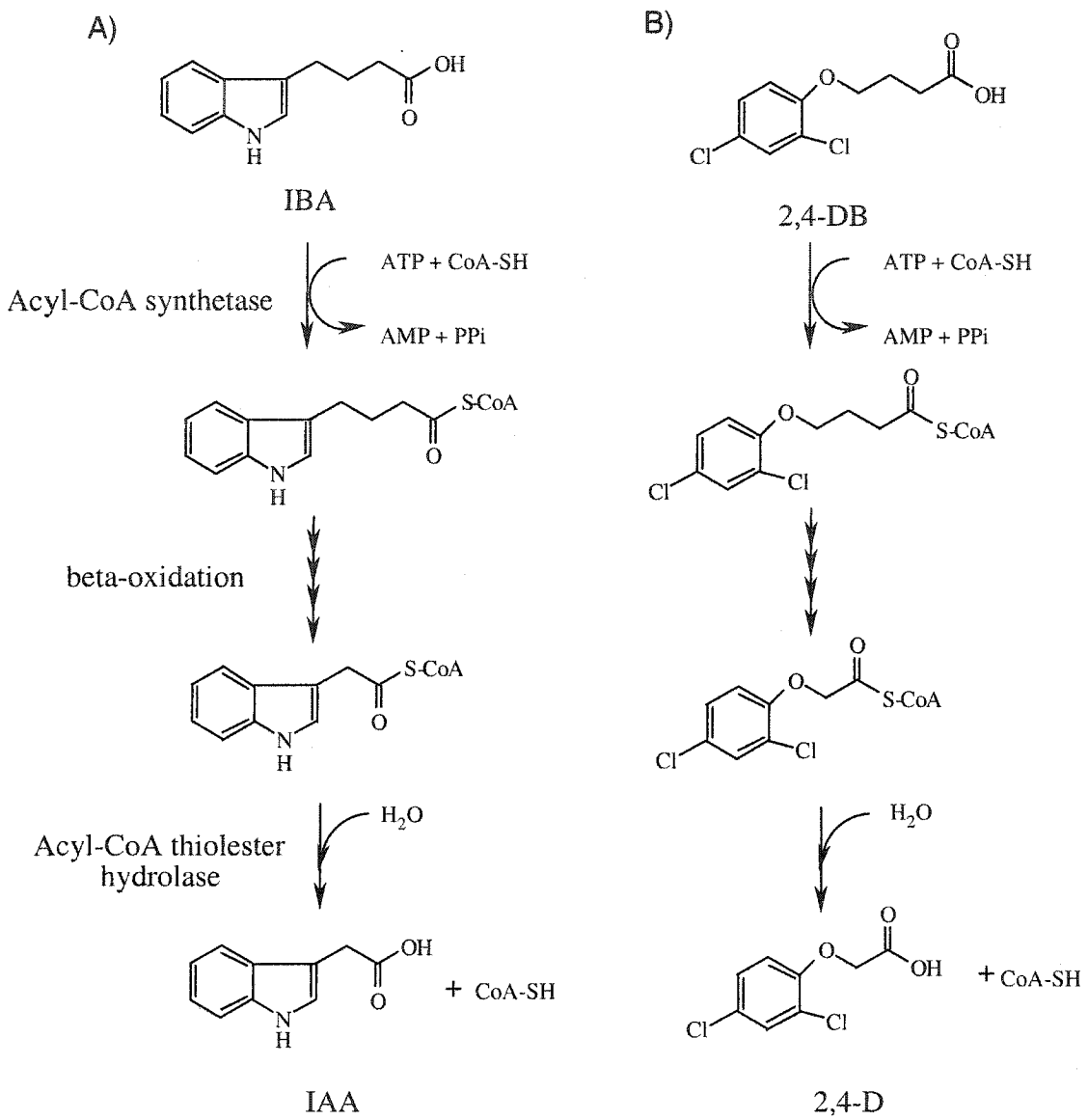

Figure 1.3: ß-oxidation of IBA and 2,4-Dichlorophenoxybutyric acid. A) IBA is converted to IAA through the chain shortening reactions of ß-oxidation. B) The inactive auxin precursor 2,4-DB is converted to the active synthetic auxin 2,4-D by ß-oxidation.

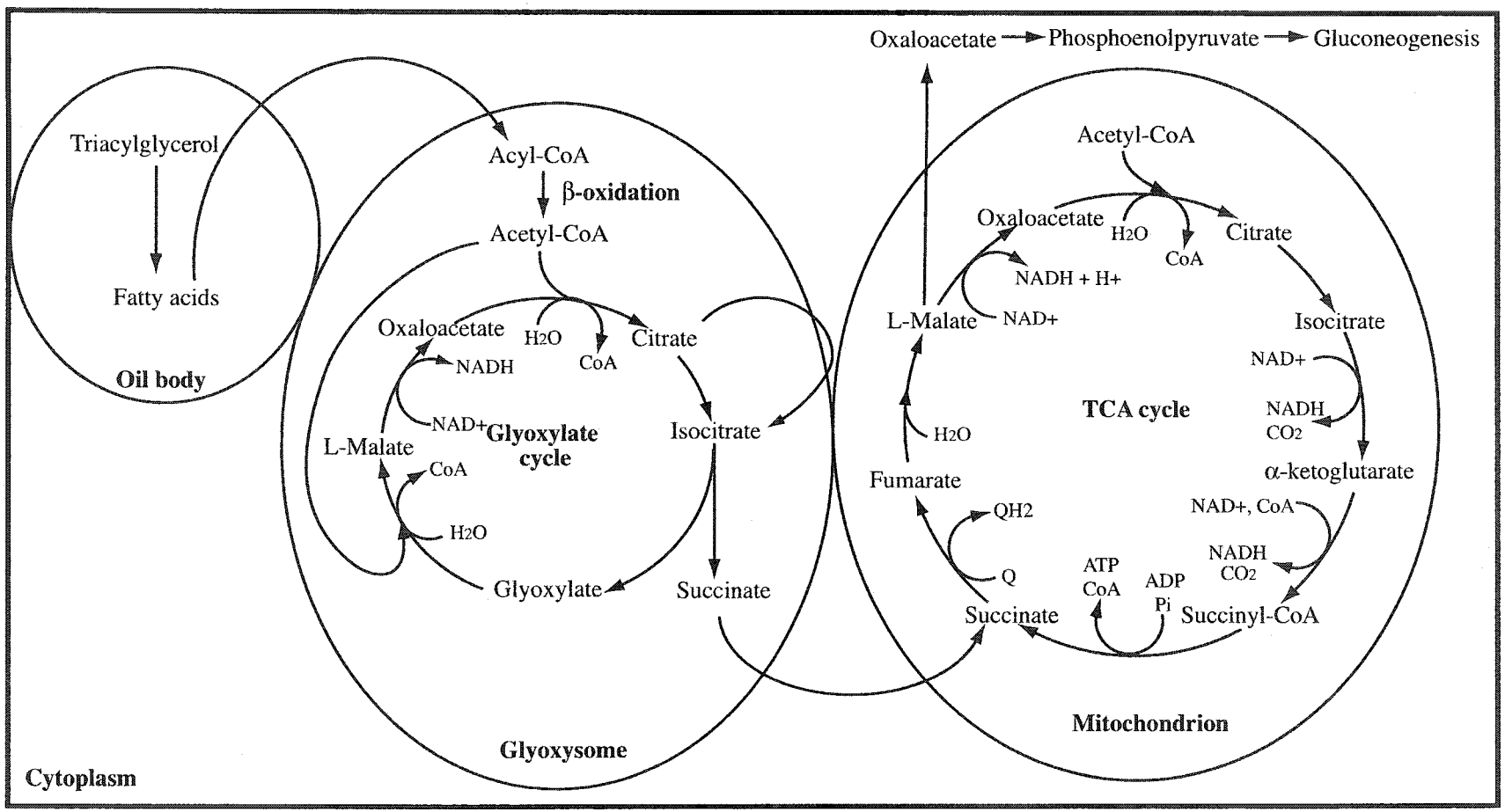

Figure 1.4: Schematic view of a plant cell showing oil reserve mobilization through ß-oxidation followed by passage through the glyoxylate cycle in the specialized peroxisomes called glyoxysomes. Succinate produced by the glyoxylate cycle is transferred to the TCA cycle in the mitochondrion. See text for details.

ß-oxidation enzymes, most enzymes of the glyoxylate cycle that uses acetyl-CoA released by ß-oxidation to produce the four-carbon molecule succinate. The glyoxylate cycle can be seen as a modified form of the tricarboxylic acid (TCA) cycle without decarboxylative steps, thereby producing carbon skeletons without net loss of carbon as CO_2 (Eastmond and Graham, 2001 – Compare the two cycles in Figure 1.4). Succinate is then transferred to the TCA cycle in the mitochondrion; malate released from the TCA cycle is then used for gluconeogenesis in the cytosol. Variations of the path to convert stored oils into usable energy exist; for example, it is thought that in certain situations acetyl-CoA or citrate from the glyoxysome could be transferred to the mitochondrion to enter the TCA cycle directly, though the glyoxylate cycle is favored during early oilseed development (Eastmond et al., 2000; Eastmond and Graham, 2001).

All peroxisomal proteins are nuclear encoded and imported post-translationally (reviewed in Johnson and Olsen, 2001, Olsen, 1998). Most, though not all, peroxisomal matrix proteins contain one of two peroxisomal targeting sequences called PTS1 and PTS2. Peroxisome biogenesis has been studied extensively in yeast, as yeast can survive without functional peroxisomes if grown on complete media. These peroxisome-deficient mutant strains have been named *peroxin (pex)* mutants (reviews of *pex* mutants can be found in Olsen, 1998 and Subramani, 1998). Many proteins involved in peroxisomal protein import have been identified through analysis of *pex* mutants. Of these, Pex5p interacts specifically with PTS1 containing proteins, and Pex7p with PTS2 containing proteins, so these are suggested to be cytosolic receptors for the PTS containing proteins. The Pex13p, Pex14p and Pex17p proteins are suggested to form a channel through which peroxisomal proteins can enter peroxisomes. Mutants in any of the channel proteins are unable to import either PTS1 or PTS2 containing proteins. It is not known how peroxisomal matrix proteins that lack PTS1 and PTS2 enter the peroxisomes. Integral membrane proteins use another signal peptide called the membrane PTS, or mPTS. Many IBA resistant mutants of Arabidopsis are affected in peroxisomal proteins, as has been revealed through cloning of the mutated genes (see Table 1.1). Zolman et al. (2000) identified mutants (class 1 mutants) that not only displayed IBA resistance, but also had a reduction in growth in the dark in the absence of sucrose, and increased levels of the fatty acid ecosenoic acid, which is an abundant Arabidopsis seed storage lipid. These

phenotypes suggest a defect in fatty acid ß-oxidation in these mutants, and three class 1 loci do encode peroxisomal proteins. One mutant line, B44, is affected in the Arabidopsis PEX5 protein, which is the peroxisomal import receptor for PTS1 harboring proteins (see above; Zolman et al., 2000). Three other IBA resistant mutants, B7, B17 and B52, all affect the same gene, *CHY1*. CHY1 is a ß-hydroxyiso-butyryl-CoA hydrolase that acts in peroxisomal valine catabolism; blocking normal valine catabolism at this step is thought to cause accumulation of the toxic intermediate methylacrylyl-CoA in peroxisomes, which can bind and inactivate coenzyme A and/or cysteine containing proteins. ß-oxidation would be blocked in this case either due to lack of coenzyme A, or by disruption of Cys containing enzymes such as thiolase (Zolman et al., 2001a). A third class 1 mutant is affected in a gene named *PXA1*. *PXA1* encodes an ATP binding cassette transporter (ABC transporter) with similarity to the human adrenoleukodystrophy (ALD) protein and other human and yeast peroxisomal transporters. This suggests PXA1 could act to import substrates, such as CoA esters of fatty acids and IBA, into peroxisomes for ß-oxidation (Zolman et al., 2001b). PXA1 alleles have been isolated in two other screens: the *peroxisome deficient 3* (*ped3*) mutant was isolated by 2,4-DB resistant growth, and *comatose* (*cts*) mutants were isolated because they are severely defective in germination (Hayashi et al., 1998; Russell et al., 2000). All *ped3* seedlings fail to establish in the absence of sucrose, and contain increased levels of lipids in 5-day-old light grown seedlings (Hayashi et al., 2002). Modification in fatty acid composition is also seen in *cts-1* seedlings. Further analysis of *cts-1* and *cts-2*, which are null alleles, has revealed that the CTS protein is important for lipid reserve mobilization and affects carbohydrate metabolism. Because a defect in acyl-CoA import cannot explain the fact that *cts-1* and *cts-2* do not germinate even in the presence of sucrose, it has been suggested that CTS, like other ABC transporters, could be a bifunctional protein. CTS could affect removal of dormancy and promotion of germination potential by a second, as yet undefined, mechanism (Footitt et al., 2002).

Other peroxisomal mutants would be expected to be resistant to IBA, and this is the case for the *aim1* and the *ped1* mutants of Arabidopsis. Richmond and Bleecker (1999) isolated a mutant named *abnormal inflorescence meristem (aim1)* that is affected in one of the two multifunctional proteins (MFP) of Arabidopsis, which catalyze steps 2

and 3 of ß-oxidation (see Figure 1.2). *aim1* has been categorized as a class 1 mutant by Zolman et al. (2000). AIM1 is a PTS1 containing protein, and it is therefore probably imported by the PEX5 protein mentioned above, which was also identified amongst the class 1 mutants. *peroxisome defective 1 (ped1)* was isolated because it is resistant to root elongation inhibition by 2,4-DB, which is converted to 2,4-D by a process similar to IBA to IAA conversion (Hayashi et al., 1998 - see Figure 1.3). *ped1* lacks the thiolase enzyme catalyzing the last step of ß-oxidation in young seedlings (step 4 of Figure 1.2). Both *ped1* and the allelic mutation *kat2* require sucrose for cotyledon expansion and seedling establishment. Loss of KAT2 results in a severely reduced capacity to metabolize stored fatty acid, and enlarged peroxisomes are seen in *ped1* and *kat2* mutants (Germain et al., 2001; Hayashi et al., 1998). *ped1* is IBA resistant.

A link between light signaling and peroxisomal function

Recently, *ted3*, a suppressor of the light response mutant *de-etiolated1 (det1)*, was shown to affect an analog of the yeast peroxisomal protein Pex2p (Hu et al., 2002). Pex2p is an integral peroxisomal membrane protein that is important for peroxisome assembly and protein import (see reviews in Olsen, 1998; Subramani, 1998). *ted3* dominantly suppresses the constitutive photomorphogenesis phenotype of *det1*, and its phenotypes are similar to those of TED3 overexpressors, indicating a hypermorphic mutation. *ted3* also partially or fully restores expression of 90% of over 900 genes affected by the *det1-1* mutation in the dark. Peroxisomal function was therefore analyzed in *det1* mutants; *det1* root elongation is resistant to IBA, and *det1-1* seedlings show several other phenotypes indicating defective peroxisomes, such as failure to establish in the absence of sucrose, morphological changes in peroxisomes and modification in expression of peroxisomal genes (see references in Table 1.1). In addition, a cross to the loss-of-function *pxa1* mutant (peroxisomal ABC transporter described above) results in synthetic lethality of the *pxa1 det1-1* double mutant. Another pleiotropic de-etiolated mutant, *cop1,* is also suppressed by *ted3*, and shows some defects in sugar and IBA response assays, suggesting peroxisomal function may be compromised in this mutant also (Hu et al., 2002).

Both COP1 and DET1 are key regulators of photomorphogenesis in plants, and are part of a group of pleiotropic mutants displaying constitutive photomorphogenesis, de-etiolated phenotypes known as the *cop/det/fus* mutants. These mutants show phenotypes of light-grown seedlings (photomorphogenesis) in the dark, such as short hypocotyls, expanded cotyledons, true leaf production and expression of light regulated genes. The recessive nature of *cop/det/fus* mutations suggests the proteins encoded by these genes act as negative regulators of photomorphogenesis, and overexpression studies with COP1 confirmed that this protein can repress photomorphogenesis in many different light conditions, in a dosage dependent fashion (McNellis et al., 1994). In the current model of light regulated development, 10 of the 11 proteins defined by *cop/det/fus* mutants are key players suggested to act by proteasome-mediated protein degradation of positive regulators of photomorphogenesis. Proteasome mediated degradation is an evolutionarily conserved mechanism through which proteins are first targeted for degradation by covalent attachment of ubiquitin by the sequential action of the E1 ubiquitin activating enzyme, the E2 ubiquitin conjugating enzyme and usually an E3 ubiquitin ligase. Ubiquitinylated proteins become substrates for the protein degradation machinery of the 26S proteasome (see also Figure 1.6C – mechanisms described in more details below). Eight members of the COP/DET/FUS family of proteins form the evolutionarily conserved COP9 signalosome, which shows similarity to the eight subunits of the lid of the 19S regulatory particle of the 26S proteasome, and could therefore act directly as the regulating portion of the proteasome. The three other COP/DET/FUS proteins are COP1, COP10 and DET1. COP1 has been suggested to act as an E3 ubiquitin ligase (Osterlund et al., 2000), but probably acts instead in collaboration with the COP1 interacting protein 8 (CIP8), which possesses E3 ubiquitin ligase activity for HY5 *in vitro* (while COP1 does not possess this activity *in vitro*) (Hardtke et al., 2002). COP10 is suggested to act as an E2 ubiquitin-conjugating enzyme (Suzuki et al., 2002). Recent publications reveal that DET1 can bind histone H2B and forms a complex with the damaged DNA binding protein (DDB1), and therefore suggest DET1 may play a direct role in regulating many genes through chromatin remodeling (Benvenuto et al., 2002; Schroeder et al., 2002).

As mentioned above, the hypermorphic peroxisomal mutant *ted3* can suppress most of the phenotypes and altered gene expression caused by both *cop1* and *det1* mutations. This suggests peroxisomes play a key role in the photomorphogenic pathway negatively regulated by DET1 and COP1 (Hu et al, 2002).

IBA resistant mutants not affected in ß-oxidation:

The function of the IBA resistant mutants of classes 2, 3 and 4 of the Zolman et al. classification is not yet known. These do not have defects in plant development in the absence of light and sucrose, and also have normal levels of the fatty acid eicosenoic acid. As they have levels of IBA resistance similar to those of class 1 mutants, they are not thought to simply be extremely leaky fatty acid ß-oxidation mutants (Zolman et al., 2000). Some of these (class 2) are defective in both root elongation and lateral root formation in response to IBA, so they could be involved in IBA to IAA conversion through a mechanism other than ß-oxidation, or they could be involved in an IBA specific signaling pathway. The single Class 3 mutant identified is resistant to IBA only for root elongation, but not for lateral root formation. This mutant (*ibr4*) therefore distinguishes these two effects of IBA on roots. Finally, members of the fourth class of IBA resistant mutants are also resistant to the synthetic auxin 2,4-D, and to auxin transport inhibitors NPA, HFCA and TIBA. These response phenotypes suggest class 4 mutants could be defective in IBA transport.

Ethylene signaling mutants

Interestingly, two independent mutants (*ein2* and *etr1*) that reduce ethylene signaling result in IBA resistant root growth. Zolman et al. (2000) found *ethylene insensitive2* (*ein2*) mutants are resistant to IBA and 2,4-D, and sensitive to IAA and NAA in root elongation assays. Null mutants at the *EIN2* locus are completely insensitive to ethylene at all concentrations and in all tissues investigated, so EIN2 is thought to be a positive regulator of ethylene signaling. Through epistasis analysis, it was determined to work downstream of ethylene receptors, but upstream of several nuclear transcription

factors (see review in Wang et al., 2002). The EIN2 protein, whose amino terminal end is similar to Nramp metal ion transporters, is proposed to act as a bifunctional signal transducer. *ein2* alleles have also been isolated as auxin transport inhibitor resistant, cytokinin resistant, delayed senescence and ABA hypersensitive mutants, and show resistance to jasmonate and paraquat (an oxygen radical releasing agent). Interestingly, none of the other ethylene-insensitive loci were isolated in such screens. It is suggested EIN2 could mediate cross talk between stress and hormone signaling pathways. Alternatively, these phenotypes may reveal a role for ethylene in mediating response to other hormones/stresses (Alonso et al., 1999, and references therein; Ghassemian et al., 2000).

The *ethylene receptor1 (etr1)* mutant is also resistant to IBA, 2,4-D and auxin transport inhibitors (Zolman et al., 2000). ETR1 is a putative ethylene receptor with similarity to prokaryote sensory transduction proteins; it is a homodimeric membrane component and binds ethylene using a copper cofactor. ETR1 was isolated as a dominant ethylene insensitive plant, and recessive loss-of-function alleles initially showed no obvious ethylene related phenotype. As five ethylene receptors exist, their function is probably at least partially redundant. The ethylene receptors are thought to be negative regulators of ethylene action, as quadruple loss-of function mutants show constitutive triple response – a phenotype associated with ethylene treatment (Hua and Meyerowitz, 1998). Cancel and Larsen (2002) recently showed that *etr1* loss-of-function alleles present a growth defect limiting plant size, and this is caused by enhanced sensitivity and exaggerated response to ethylene, indicating other receptors are not fully redundant with ETR1.

Cross talk between hormone signaling pathways is a recurring theme in plant physiology. For example, root growth inhibition by ABA requires a functional ethylene signaling cascade, as *etr1* and *ein2* mutants abolish this inhibition (Beaudoin et al., 2000). Specific interactions between ethylene and auxin signaling cascades have also been revealed through the years. Ethylene can inhibit auxin transport (Beyer, 1973), and *eir1*, a mutant isolated as having ethylene insensitive roots, is affected in a gene encoding a putative auxin efflux carrier (Luschnig et al., 1998). Auxins induce certain isoforms of the first enzyme of ethylene biosynthesis, named ACC synthase (ACS) (Wang et al.,

2002), and many auxin resistant mutants are also resistant to a number of other hormones including ethylene (see Tables 1.2 and 1.3 and references therein), revealing further cross talk between auxin and ethylene signaling pathways. Exactly how a reduction in ethylene signaling could result in IBA resistance is not clear at present. Ethylene could affect IBA distribution through its effect on transport, or IBA could induce ethylene synthesis, or yet another, uncharacterized type of interaction could occur between the two signaling pathways.

The *rib1* mutant

In the next chapter, I present the characterization of the *resistant to IBA* (*rib1*) mutant, whose phenotypic characterization suggests it is affected in IBA response or transport. In order to better understand the role and mechanism of action of IBA, and since the phenotypes of *rib1* described in Chapter 2 could be consistent with a defect in IBA transport, it was important for us to first investigate IBA transport in wild-type plants and then to determine whether this wild-type pattern of IBA transport was modified in *rib1*. These experiments are the subject of Chapters 3 and 4, respectively.

Auxin transport in plants:

The overwhelming majority of studies of auxin transport in plants have focused on IAA and a relatively clear picture of the transport of this auxin has therefore emerged in recent years. The following section presents an overview of how IAA is transported in plants. The introduction to Chapter 3 details what is known about IBA transport.

IAA transport

The auxin IAA undergoes highly regulated polar transport from its point of synthesis in apical portions of the shoot towards the rest of the plant. Three flows of IAA transport have been described in plants: from the young leaves and meristems that produce IAA to the base of the plant (basipetal shoot or hypocotyl transport), then from

the base of the root towards the root tip (root acropetal transport) and finally from the root tip back up a short distance to the root elongation zone (root basipetal transport - see Figure 1.5 A - Davies and Mitchell, 1972; Rashotte et al., 2000; Rashotte et al., 2001). These different flows of IAA have been suggested to be important for elongation responses, lateral root development and gravitropic responses in roots, respectively (Casimiro et al., 2001; Muday and Haworth, 1994; Rashotte et al., 2000; Reed et al., 1998b). Auxin transport has also been shown to be important for proper initiation and positioning of shoot lateral organs and establishment of bilateral symmetry of embryos (Liu et al., 1993; Reinhardt et al., 2000)

The chemiosmotic model explains how transport polarity is achieved (Figure 1.5B -see reviews in Goldsmith, 1977; Lomax et al., 1995). This model depends on the difference in pH between the apoplastic space (cell walls – acidic pH of approx. 5.5) and the interior of cells (cytoplasm, neutral pH approx. 7.0), and the changes in ionization state of IAA that occurs at these different pH values. IAA in the acidic conditions found outside cells, is coupled to a proton (H+) and therefore has a neutral charge making it capable of entering cells passively through the cell membranes. Auxin can also be actively pumped inside cells by auxin influx carriers. Once inside the cell, at neutral pH, IAA dissociates from its proton and is found in a negatively charged form that is polar and therefore cannot pass through the lipophilic cell membrane without the help of a carrier. The aptly named auxin efflux carrier is located at the base of cells (in stems), so IAA can only exit cells at this location, and therefore can only move towards the cell below. Basal localization of efflux carriers is the key element leading to polarity of transport. Polar auxin transport is an energy requiring process, as it can be blocked by anaerobiosis and respiration inhibitors (Lomax et al., 1995); energy is also required to maintain the pH by active H+ excretion, and to maintain membrane potential and the polar localization of efflux carriers (Goldsmith, 1977).

This chemiosmotic model for auxin transport, proposed independently by Rubery and Sheldrake (1974) and by Raven (1975) based on important physiological studies, has been confirmed in recent years and somewhat extended by molecular and genetic studies. Notably, genes encoding the putative influx and efflux carriers have been identified, and shown to be members of large gene families. Analysis of protein localization confirmed

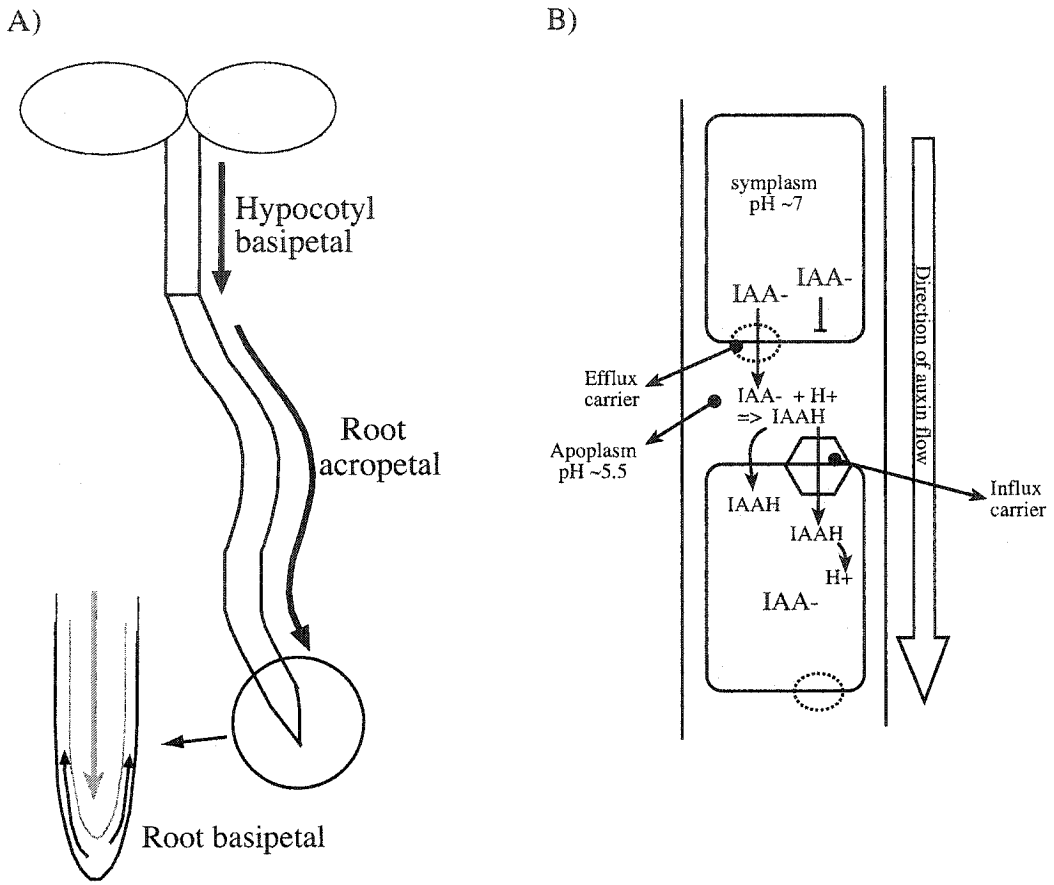

Figure 1.5: Polar auxin transport in Arabidopsis and other plants.
A) The different flows of IAA in seedlings. IAA produced in apical portions of the seedling are transported from the tip of the seedling to the base of the hypocotyl (hypocotyl basipetal transport), and then from the base of the root towards the tip through the central tissues of the root (root acropetal transport). Finally IAA at the root tip move back towards the base of the root through the epidermal tissue for a few millimeters (root basipetal transport - circle representing magnified root tip region shown left.)
B) Model for polar cell-to-cell auxin transport. This model depends on a pH difference between the interior of the cell (symplasm - pH 7.0) and the cell walls surrounding the cells (apoplasm - pH 5.5). Inside the cell, IAA is in an ionized form that cannot exit the cell unless a specific transporter (represented by an oval), the efflux carrier, is present. In the apoplasm, where the pH is lower, IAA is mainly found in a protonated form, which is more lipophilic and can therefore go through the cell membrane to enter the next cell. Additionally, an IAA influx carrier (represented by an hexagon) can facilitate IAA upload into the next cell. Polar localization of efflux carriers at one end of cells in files of cells result in directionality of transport. Note that multiple types of influx and efflux carriers exist.

that efflux carriers are polarly localized to one end of cells. In addition, different carriers can be found in specific cells in appropriate locations to mediate the different flows of IAA described in Figure 1.5A. Phenotypic analysis of auxin transport mutants in Arabidopsis have confirmed the importance of polar transport for normal flower and root development, for response to environmental stimuli such as light and gravity and for vascular tissue formation (see Table 1.2 at the end of this section).

Regulatory molecules and proteins that modulate IAA transport have been identified through physiological and genetic studies (see recent review by Muday and DeLong, 2001). Inhibitors of IAA transport exist: NPA (naphthylphthalamic acid - a phytotropin), TIBA (triiodobenzoic acid) and HFCA (9-hydroxyfluorene-9-carboxylic acid - a morphactin) have been shown to block efflux of IAA from cells and are used routinely as inhibitors of IAA efflux in physiological studies. As these efflux inhibitor molecules have different molecular configurations and response profiles, they are proposed to have different modes of action (Katekar and Geissler, 1980; Rubery, 1990). Recent studies suggest NPA could act by affecting the sub-cellular localization of efflux carriers (Geldner et al., 2001). Endogenous molecules thought to act as modulators of auxin transport include flavonoids, such as quercetin and kaempferol, linoleic acid and a number of proteins, such as the PINOID Ser/Thr kinase and the RCN1 protein phosphatase 2A regulatory subunit A, implicating reversible protein phosphorylation in control of auxin transport (Benjamins et al., 2001; Brown et al., 2001; Christensen et al., 2000; Muday and DeLong, 2001; Murphy et al., 2000; Rashotte et al., 2001; Suttle, 1997).

The auxin transport mutants *aux1, axr4, eir1* and *tir2*

Genetic analysis has revealed a number of genes whose disruption causes phenotypes suggestive of defects in auxin transport, and many of these are described in Table 1.2. Others are discussed in more detail in the introductions to the manuscripts of Chapters 3 and 4. I will review here the phenotypes of a subset of auxin transport mutants that are of particular interest as these were used in double mutant studies

presented in Appendix 1. The phenotypes of *aux1, axr4, eir1* and *tir2* single mutants are presented with the current model for the function of the affected gene.

The phenotypes of *aux1* mutants include auxin resistant and agravitropic root growth (see Table 1.2 and references therein). Lateral root formation is decreased and root growth is also resistant to ethylene and BA. Apart from these defects in root growth and response, *aux1* plants resemble wild-type. The *AUX1* gene was cloned and encodes a protein with similarity to amino acid permease and is proposed to act as an auxin (IAA) influx carrier (Bennett et al., 1996). As amino acid permeases are proton driven symporters, this similarity suggests IAA uptake would also be proton driven. Promoter fusion studies show AUX1 is expressed in the shoot apical meristem, in the primary root, and throughout lateral root development (Marchant et al., 2002). Analysis of IAA distribution in wild-type and *aux1* mutants shows differences that can be explained by a model in which AUX1 has a role in loading IAA in the phloem from leaves and unloading at the root tip, thereby regulating root initiation and emergence. The lipophilic synthetic auxin NAA, which is thought to enter cells without a need for an influx carrier, can rescue the reduction in lateral root phenotype of *aux1*, confirming auxin levels in the root apex are suboptimal for lateral root formation (Marchant et al., 2002).

axr4 was the first mutant isolated that is specifically resistant only to auxins, and not to other classes of plant hormones (Hobbie and Estelle, 1995). *axr4* also shows phenotypes consistent with a defect in auxin response or transport such as reduced root gravitropism, increased root elongation and a reduction in the number of lateral roots (Table 1.2 and references therein). Another allele of *axr4* was isolated in a screen for root gravitropic mutants and named *rgr1* (*reduced root gravitropism1*). Root elongation in this mutant is resistant to inhibition by auxin transport inhibitors, and more resistant to IAA (6-fold) than to 2,4-D (3.3-fold), or NAA (1.8-fold) (Simmons et al., 1995a). Hypocotyl elongation is also 2,4-D resistant. Other authors have found *axr4* roots to have a wild-type response to NAA, and show that NAA can rescue the agravitropic nature of *axr4* root growth (Yamamoto and Yamamoto, 1999). As NAA is thought to enter cells by diffusion and not through auxin influx carriers, these results suggest *axr4* could be defective in an auxin influx carrier.

eir1 was isolated through a screen for mutants resistant to growth inhibition by ethylene (Roman et al., 1995). It was later shown to be allelic to mutations isolated through screens for mutants with defects in root gravitropism (*agr1*) and wavy root growth (*wav6*) (Bell and Maher, 1990; Luschnig et al., 1998; Okada and Shimura, 1990). The defects in these mutants, limited to roots, affect elongation and gravitropism, and response to auxins, the ethylene precursor ACC and the IAA transport inhibitor TIBA. Cloning of the *AGR1/EIR1/WAV6* gene showed these allelic mutations lie in the *PIN2* gene, so-called because it belongs to a family of eight putative auxin efflux carriers in Arabidopsis homologous to *PIN1* (Chen et al., 1998; Luschnig et al., 1998; Müller et al., 1998; Utsuno et al., 1998). *PIN* gene products are integral membrane proteins thought to act as auxin efflux carriers. *agr1* mutants were also shown to have reduced IAA efflux, supporting the hypothesis that the affected gene encodes an efflux carrier (Chen et al., 1998).

tir2 was isolated in a screen for mutants resistant to the IAA transport inhibitors NPA, 2-carboxyphenyl-3-phenylpropane-1,2-dione (CPD) and methyl-2-chloro-9-hydroxyfluorene-9-carboxylate (CFM) (Ruegger et al., 1997). *tir2* displays normal sensitivity to auxins but differs from wild-type for lateral root formation, hypocotyl length and root hair number (Jensen et al., 1997). These phenotypes suggest a defect in auxin transport in this mutant.

In addition to investigating interactions between *rib1* and the IAA transport mutants described above, we also generated double mutants with mutations in genes involved in auxin signaling/response (*axr1, axr2*), in regulation of auxin levels (*rty1*) and in light and auxin regulated development (*hy5*). The results of these analyses are presented in Appendix I. This approach was used as a first step to further investigate cross-talk between IBA, IAA and light signaling pathways, suggested to occur based on the results presented in Chapter 2, 3 and 4. In the following paragraphs, I overview the current model for IAA signaling, and introduce the auxin signaling mutants used in double mutant analysis. A brief description of the gene product affected and of the phenotypes of auxin resistant and selected response mutants can also be found in Table 1.3, presented at the end of this introduction.

Auxin signaling:

Auxins affect nearly all aspects of plant growth, from embryogenesis to flowering. One question that has long puzzled plant physiologists is how a simple molecule like IAA could have such ubiquitous roles, and more specifically, how the same hormone could affect virtually all parts of the plant and result in different and appropriate responses from each one. Part of the answer to this question has been found in recent years through genetic analysis of auxin response. This analysis has revealed the existence of numerous proteins involved in auxin transport and response, and results in a model for auxin response such as the one presented in Figure 1.6 (see reviews by Hellmann and Estelle, 2002; Leyser, 2002, and references therein). The Aux/IAA proteins are of central importance to auxin signaling. The first members of this family of proteins were identified based on their rapid induction by auxins, and were therefore thought to be primary auxin response genes (see recent review by Reed, 2001). Many other Aux/IAA proteins have been identified by sequence similarity to the initial proteins, and it is estimated that the Aux/IAA family comprises 25 genes, and is probably unique to plants (Reed, 2001). Canonical Aux/IAA proteins contain four conserved sequence motifs, designated domains I, II, III and IV. Domain I is the least conserved of these, and no biochemical function has been ascribed to it. Domain II is thought to be important in regulating Aux/IAA protein levels by destabilizing the proteins (Ramos et al., 2001; see below), and domains III and IV are dimerization domains that allow formation of homo- and heterodimers. Aux/IAA proteins can form heterodimers with each other, but also with another important class of proteins, the so-called auxin response factors (ARF). ARF proteins are another large family of proteins (23 members) whose COOH-terminal ends contain the conserved domains III and IV of Aux/IAA proteins, allowing ARFs to homo- and heterodimerize with each other and with Aux/IAA proteins. In addition, ARFs contain a DNA binding domain, and a more variable central region that allows them to either activate or inhibit gene transcription following interaction with auxin response elements (ARE) found in the promoter region of auxin regulated genes (Ulmasov et al., 1997; 1999a; 1999b). Truncated versions of Aux/IAA and ARF proteins

Figure 1.6: Brief overview of the current auxin signaling model (see figure legend next page)

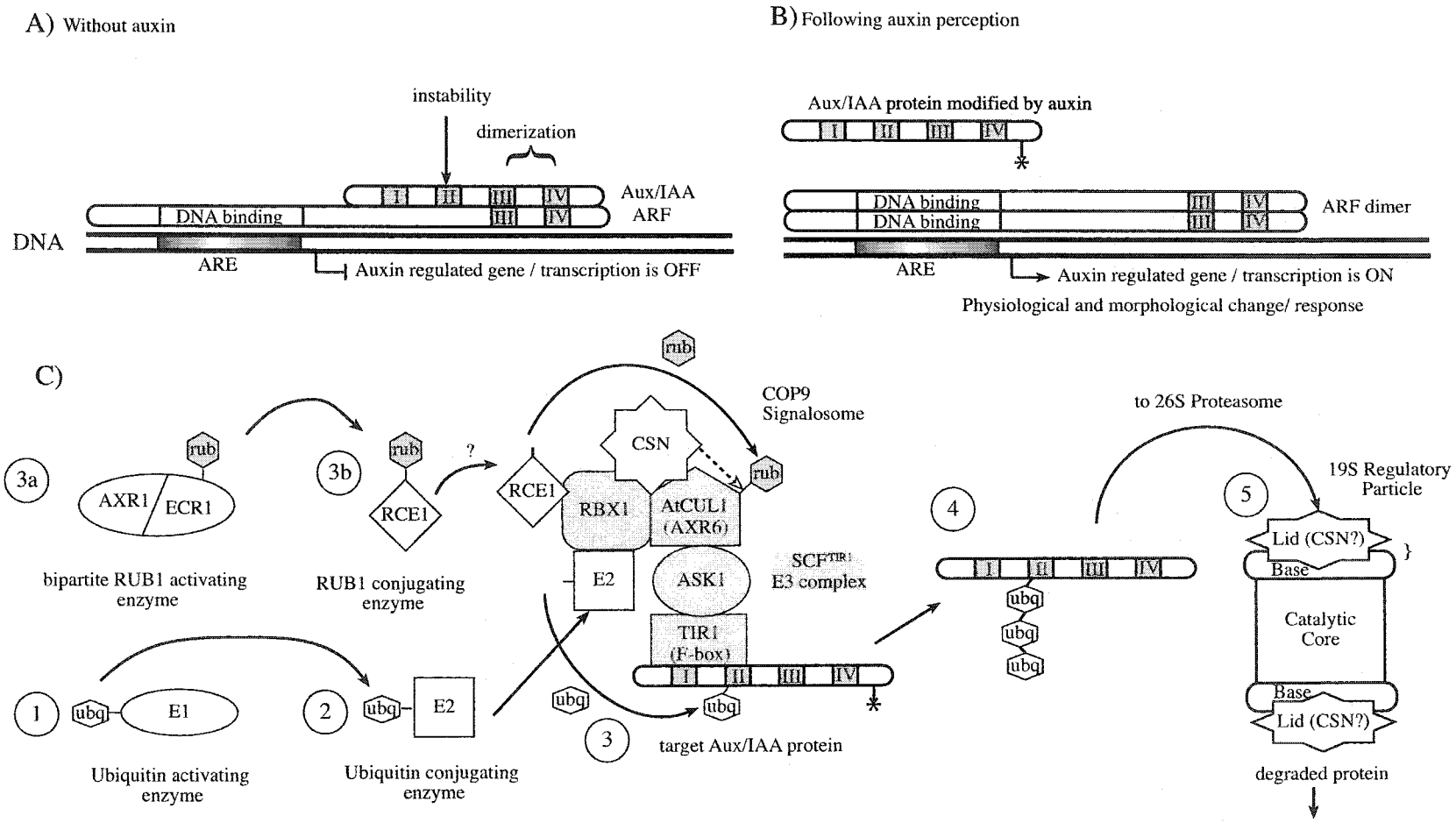

Figure 1.6, legend:

Simplified model of how Aux/IAA and ARF proteins can positively regulate gene transcription in response to auxin. (Note that certain ARF proteins also repress gene expression – please see text). Domains III and IV of Aux/IAA proteins are important for dimerization with other Aux/IAA proteins, and with proteins called Auxin Response Factors (ARFs). ARFs share domains III and IV with Aux/IAA proteins, but also contain a DNA binding domain that allows them to activate transcription of genes whose promoter regions contain sequences known as Auxin Response Elements (ARE – shaded boxes on DNA). In the absence of auxin (A), ARF proteins are bound to ARE of auxin-regulated genes, but dimerization with an Aux/IAA protein prevents transcription of the gene. Perception of auxin (B) presumably produces a change in the Aux/IAA (represented by a bound asterisk) that releases it from the promoter-bound ARF, allowing dimerization of the ARF with another ARF. The ARF dimer produced can activate gene expression from the ARE containing promoter. The nature of the modification of the Aux/IAA protein is as yet unknown, but it increases the affinity of the Aux/IAA protein for binding to an enzyme complex know as the SCF^{TIR1}, involved in ubiquitination and eventually proteolysis (depicted in light blue in panel C). The increased affinity of Aux/IAA proteins for a degradative pathway presumably changes the equilibrium of dimerization from a point where most ARFs are dimerized with Aux/IAA proteins, to a point where most ARFs are dimerized with other ARFs. C) Ubiquitin conjugation and proteolysis of Aux/IAA proteins is a multi-step process. 1- the small protein ubiquitin (ubq) is activated by interaction with an E1 ubiquitin activating enzyme. 2- ubq is transferred to an E2 ubiquitin conjugating enzyme. 3- with the help of a protein complex called the E3 ubiquitin ligase, ubiquitin is transferred from the E2 to the target Aux/IAA protein. The E3 complex involved in auxin response is called SCF^{TIR1}, and is composed of a SKP-like protein (ASK1), a cullin (AtCUL1/AXR6), an F-box protein (TIR1), and a RING H2 protein called RBX1. The TIR1 protein binds the protein targeted for ubiquitination, while the RBX1 protein directly interacts with the E2 allowing transfer of ubq from the E2 to the target (in this case the Aux/IAA protein). Optimal E3 activity depends on modification of the cullin portion of the complex by conjugation to another

Figure 1.6 legend - continued

small protein called Related to Ubiquitin, or RUB. 3a – RUB is activated by interaction with a bipartite E1-like enzyme made of AXR1 and ECR1 proteins. 3b – RUB is transferred to a RUB conjugation enzyme (RCE1). RCE1 probably also interacts with RBX1 of the E3 complex and RUB is transferred to AtCUL1/AXR6. Note that another protein complex, the COP9 signalosome, can interact with the E3 complex and remove RUB from the Cullin (dashed arrow). 4- Multiple ubq molecules can be added to the target by repetition of the cycle described above, and ubiquitin chain elongating enzymes also exist. 5- Polyubiquitinylated proteins are targets for degradation by the 26S proteasome. The COP9 signalosome has similarity to the lid portion of 19S regulatory protein that is part of the proteasome, and has therefore been suggested to act as an alternative lid. The regulatory particle is important in selecting targets for degradation. Alternatively or concurrently, auxin has also been suggested to act by modifying the activation state of the SCF^{TIR1}. Please refer to text for more details.

also exist that, depending on the domains they contain, can have activity by competing for dimerization or binding to AREs. A current simplified model of how Aux/IAA and ARF proteins can positively regulate gene transcription in response to auxin is depicted in panels A and B of Figure 1.6 (see also Figure 1 of Leyser, 2002). In the absence of auxin (Fig. 1.6A), ARF proteins are bound to the ARE of auxin-regulated genes, but dimerization with an Aux/IAA protein prevents transcription of the gene. Perception of auxin (Fig. 1.6 B) presumably produces a change in the Aux/IAA that releases it from the promoter-bound ARF, allowing dimerization of the ARF with another ARF. The resulting ARF dimer can activate gene expression from the ARE containing promoter. The nature of the modification of the Aux/IAA protein is as yet unknown, but it increases the affinity of the Aux/IAA protein for binding to an enzyme complex known as the SCF^{TIR1} E3 complex, involved in ubiquitination and eventually proteolysis (Figure 1.6C) (Gray et al., 2001). Alternatively or concurrently, auxin has also been suggested to act by modifying the activation state of the SCF^{TIR1} E3 complex (Zenser et al., 2001; see below). The increased affinity of Aux/IAA proteins for a degradative pathway presumably changes the equilibrium of dimerization from a point where most ARFs are dimerized with Aux/IAA proteins, to a point where most ARFs are dimerized with other ARFs. This simple model is however further complicated by the fact that many Aux/IAA proteins themselves can be induced by auxin, that some ARFs have inhibitory rather than stimulatory effects on transcription of ARE containing genes (Ulmasov et al., 1999a), and that Aux/IAA proteins can also be modified by signals other than auxin, notably the light receptor phytochrome (Colon-Carmona et al., 2000). Precise analysis of this system is also complicated by the sheer number of possible interacting partners, and possible redundancy in their function: 23 ARFs can interact with themselves and each other and with 25 Aux/IAA proteins which, in turn, can also homo- and hetero-dimerize.

Many components of the ubiquitin degradative pathway shown in Figure 1.6C have been identified in recent years (see also models by Hellmann and Estelle, 2002; Leyser, 2002; Rogg and Bartel, 2001). Ubiquitin is a small protein, found in nearly all eukaryotes, which binds to proteins and targets them for degradation by the 26S proteasome in a three step process. Ubiquitin is first activated by binding to the E1 ubiquitin activating enzyme, it is then transferred to the E2 ubiquitin conjugating enzyme,

and the E2, together with a third player, the E3 ubiquitin ligase, transfers ubiquitin to the targeted protein (Scheffner et al., 1995). Substrate specificity of these reactions depends on the E3 enzyme, and different forms of E3 enzymes exist in eukaryotes (Callis and Vierstra, 2000). Aux/IAA proteins are ubiquitinated through a three step process involving an E1, an E2 and an E3 as described above. The E3 involved in auxin mediated Aux/IAA protein degradation is of the multimeric SCF type. The SCF E3 complex consists of at least three subunits: One with similarity to Skp, one that is a member of the Cullin family of proteins, and one F-box containing protein (Gray et al., 1999). The F-box protein specifies the target protein that will be ubiquitinated; in the case described here, the F-box protein responsible for binding Aux/IAA protein is called TIR1, so the E3 complex is called SCF^{TIR1}. Optimal E3 activity also requires modification of the Cullin part of SCF^{TIR1} (AtCUL1) by addition of a small RUB protein (Related to UBiquitin –called Nedd8 in other species). Addition of RUB also occurs in a sequential manner similar to that of ubiquitin; in this case a bipartite protein made of AXR1 and ECR1 proteins acts in a similar fashion as E1 to activate RUB1 (del Pozo et al., 1998), which is then transferred to a RUB Conjugating Enzyme (RCE1), and finally, following interaction of the RCE1 with a fourth component of the SCF^{TIR1}, RBX1, RUB is transferred to the cullin AtCUL1 (del Pozo et al., 2002; Gray et al., 2002). The COP9 signalosome (CSN) interacts with the SCF^{TIR1} (Schwechheimer et al., 2001) and is also involved in RUB modification; this large protein complex is suggested to remove RUB from AtCUL1, thereby regulating SCF^{TIR1} activity (Lyapina et al., 2001). In addition to possibly causing a direct modification of Aux/IAA proteins, auxin has also been proposed to increase Aux/IAA proteolysis by altering the SCF^{TIR1} activation state through RUB addition, or by altering CSN activity or interaction with SCF^{TIR1} (Zenser et al., 2001). The final step of ubiquitination is transfer of ubiquitin from the E2 interacting with the E3 (activated by RUB conjugation) to the Aux/IAA protein. This process can be repeated and ubiquitin chain elongating proteins also exist, so that target proteins are polyubiquitinated. Polyubiquitinated proteins are then degraded by the 26S proteasome. The 20S proteasome catalytic core is normally complexed with a 19S regulatory protein, composed of a lid and a base, thought to act in target selection. Interestingly, the COP9 signalosome shows similarity to the lid particle, and has therefore been suggested to act

as a lid particle to select targets for degradation (see reviews by Hardtke and Deng, 2000; Schwechheimer and Deng, 2000).

The *axr1-3* mutant used in this study was isolated from a screen of EMS generated mutants for 2,4-D resistant root elongation (Estelle and Somerville, 1987). The *axr1-3* mutation affects growth of almost all plant organs, including hypocotyl elongation in the dark, shoot apical dominance, root gravitropic response, lateral root formation and elongation. *axr1-3* mutants also have reduced fertility, flowers with short stamens, and smaller leaves (see Table 1.3 for more detailed phenotypic description and references). *axr1-3* root growth is resistant to auxins, but also to the cytokinin benzyladenine (BA) and to ethylene. Another allele of *axr1*, *axr1-24*, that was isolated by resistance to Methyl Jasmonate (MeJa), also shows resistance to the ethylene precursor ACC, epibrassinolide and ABA. *axr1* mutants therefore display numerous pleiotropic growth and response phenotypes.

The *AXR1* gene was cloned and encodes a protein with similarity to the amino-terminal half of the ubiquitin-activating enzyme E1, and, as described above (see Figure 1.6) acts to activate RUB. AXR1 was the first protein with similarity to only one half of the E1 enzyme to be identified (Leyser et al., 1993). It was shown to function as a RUB activating enzyme when combined with the ECR1 protein, which resembles the C-terminal half of canonical full-size E1 enzymes. AXR1 protein function is necessary for RUB modification of the cullin subunit of SCF^{TIR1} (del Pozo and Estelle, 1999), but has also been suggested to be important for the function of other SCF type E3 enzymes, such as those involved in jasmonic acid, cold and light signaling (Schwechheimer et al., 2002).

Another auxin signaling mutant used to generate double mutants in this study is *axr2-1*. This mutant was isolated in a screen for IAA resistant mutants (Wilson et al., 1990). The phenotypes of the *axr2* mutants, as described in Table 1.3, include auxin resistant root and hypocotyl elongation, dwarfism, and defects in shoot and root gravitropism (see references in Table 1.3). In both the light and the dark, *axr2* hypocotyls are shorter than wild-type. The difference is more dramatic in the dark, where *axr2* causes partial photomorphogenesis, including short hypocotyls, open cotyledons and production of true leaves. These phenotypes are characteristic of light-grown seedlings, and are not seen in dark-grown wild-type seedlings. Lateral root formation and root

elongation can be stimulated by exogenous IAA (and IBA for lateral root formation), indicating that roots retain sensitivity to auxins in the *axr2* background. As IAA has very little effect on the hypocotyl phenotypes of *axr2* seedlings, it has been suggested that the AXR2 protein is more important for auxin response in hypocotyls than roots.

The *axr2* mutations lie in the IAA7 gene (Nagpal et al., 2000). The *axr2-1* allele used in this study is a dominant gain-of-function one. It affects the highly conserved domain II of the protein, stabilizes the IAA7 protein causing its misexpression and increased activity. Many other mutations in domain II of *Aux/IAA* genes result in similar gain-of function stabilization of the proteins, while addition of domain II to unrelated proteins causes instability. This domain is therefore suggested to be important for control of Aux/IAA protein levels *in vivo* (Reed, 2001).

The *rooty1-5* mutation results in increased auxin accumulation

In addition to mutants affected in auxin response and transport, we also investigated the interactions between *rib1* and the *rty1* mutant. Mutants at the *RTY1* locus have been isolated from different laboratories, and are known under different names: *aberrant lateral root formation* (*alf1*) (Celenza et al., 1995), *superroot1* (*sur1*) (Boerjan et al., 1995), *invasive root* (isolated by ZR Sung, UC Berkeley), *rooty1-1* (*rty1-1* King et al., 1995), *hookless3* (*hls3*) (Lehman et al., 1996), *rty1-3* (Gopalraj et al., 1996) and *rty1-5* (Windsor 2001). *rty1* mutants have short hypocotyls and no apical hook formation in the dark, dramatic increases in lateral and adventitious root formation, epinastic cotyledons and leaves, and are infertile because they fail to produce an inflorescence. These phenotypes can be phenocopied by growth of wild-type plants on high levels of auxin, and are associated with elevated levels of endogenous auxin in the mutants (Celenza et al., 1995; King et al., 1995). RTY encodes a protein with similarity to tryptophan aminotransferases (Gopalraj et al., 1996), possibly involved in IAA biosynthesis from tryptophan, or in regulating IAA levels (Bartel, 1997).

The long hypocotyl mutant *hy5*

As the *rib1* mutant displays a long hypocotyl under certain growth conditions (see Chapter 3), we generated double mutants of *rib1* with the *hy5* mutant. The *hy5* mutant is unique amongst the long hypocotyl (*hy*) family of mutants because it displays a long hypocotyl phenotype in red, far-red and blue light (Koornneef et al., 1980), implying it is involved in transduction of light signals from multiple photoreceptors, while other *hy* mutants show a long hypocotyl phenotype only under certain light conditions (Koornneef et al., 1980). *hy5* mutants also display phenotypes reminiscent of those seen in auxin resistant mutants, including defects in primary and lateral root gravitropism, increased number of lateral root primordia and increased lateral root elongation (Oyama et al., 1997). In addition, *hy5* mutants have decreased secondary thickening of roots and hypocotyls, and reduced greening of hypocotyls and roots in the light when compared to wild-type (Oyama et al., 1997). The *HY5* gene was cloned and the carboxy-terminal end of the protein is homologous to the DNA binding and dimerization domain of bZIP proteins (Oyama et al., 1997). Its capacity to bind DNA suggests HY5 could be a transcriptional regulator, and HY5 has in fact been shown to bind and activate transcription from the promoter of the light regulated *CHALCONE SYNTHASE* (*CHS*) gene (Ang et al., 1998). The HY5 protein also contains a leucine zipper motif and conserved casein kinase II (CKII) phosphorylation sites (Oyama et al., 1997). Phosphorylation of the protein was shown to affect its activity, stability and ability to interact with other proteins, notably COP1 (Hardtke et al., 2000). HY5 is hypothesized to be a positive regulator of photomorphogenesis whose levels and activity is regulated by light (Osterlund et al., 2000) and by interaction with other proteins, notably the HY5 homolog HYH, and members of the COP/DET/FUS family of proteins. Specifically, HY5 directly interacts with the COP1 protein, and this light dependent interaction results in negative regulation of HY5. As discussed above, there are 11 *COP/DET/FUS* loci, and mutations at these result in phenotypes of recessive constitutive photomorphogenesis. The proteins encoded by these genes are therefore suggested to be negative regulators of photomorphogenesis (Hardtke and Deng, 2000; Schwechheimer and Deng, 2001). The COP9 signalosome (CSN) is made of 8 different COP and FUS proteins, while COP10, COP1 and DET1 are not subunits of the CSN. COP10 encodes an E2 enzyme variant and

the COP1 interacting protein CIP8 shows ubiquitin ligase (E3) activity; these proteins are thought to mediate ubiquitinylation and degradation of HY5 and the HY5 homolog HYH (Hardtke et al., 2002; Suzuki et al., 2002).

Thesis outline: rationale and objectives

The main goal of this thesis was to understand more thoroughly the possible function and mode of action of the auxin IBA in the model plant Arabidopsis. As mentioned previously, at the time this work was initiated very few studies had focused on this auxin, yet it is present at physiologically active concentrations in many plant species, including *Arabidopsis thaliana*. In Chapter 2, I present the isolation and characterization of the *rib1* mutant, which is amongst the first mutants to be specifically resistant to IBA while retaining wild-type sensitivity to IAA. My first objective was to use this mutant to genetically dissect the role of IBA from that of IAA by studying the phenotypes caused by a defect in IBA response. Many of the phenotypes uncovered during this study were consistent with a possible defect in IBA transport in the *rib1* mutant. As IBA transport in Arabidopsis seedlings and adult plants had not been described, we analyzed IBA transport and specifically compared it to IAA transport, which is well characterized in Arabidopsis, both physiologically and genetically. The analysis of IBA transport in wild-type Arabidopsis plants is the subject of Chapter 3. In Chapter 4, we pursue our analysis of IBA transport, but look at transport in a *rib1* mutant background, and find that IBA transport is altered. Finally, in Chapter 5, I review the results presented in the previous chapter and draw conclusions based on these results as to the possible function of the RIB1 protein, and the role and mode of action of IBA in Arabidopsis. Preliminary analysis of double mutants of *rib1* with mutants in auxin response or transport, regulation of auxin levels or regulation of hypocotyl elongation is presented in Appendix I, along with future prospects for analysis of these double mutants. These analyses could result in a more thorough understanding of the role or interactions of RIB1 with well-defined auxin or light response pathways.

Table 1.1: IBA resistant mutants of Arabidopsis

Mutant / gene name (symbol) ***Aliases (Allelic gene names)*** **Proposed protein function**[1]	**Mutant phenotypes**[2]
Peroxisomal / ß-oxidation mutants	
CoA-ester hydrolase 1 (chy1) β-hydroxyiso-butyryl-CoA hydrolase: Acts in peroxisomal valine metabolism	• *chy1-1* (B17), *chy1-2* (B52) and *chy1-3* (B7) isolated as Bartel lab Class 1 mutants, with (putative) ß-oxidation defects: IBA resistant, IAA sensitive root elongation and lateral root formation. Reduced hypocotyl elongation in the dark in the absence of sucrose and increased levels of the fatty acid eicosenoic acid. Resistant to 2,4-DB also, but not to 2,4-D or NPA. (Zolman et al., 2000) • *chy1* responds like WT to 2,4-D, NAA, ACC, benzyladenine. *chy1* is recessive for IBA resistance. Fewer lateral roots in the absence of auxin and in response to IBA, but WT numbers of lateral roots formed on IAA. The short hypocotyl in the dark in the absence of sucrose and IBA resistance phenotypes of *chy1* can be rescued by human homolog of CHY if targeted to peroxisomes. (Zolman et al., 2001a)
B11	Class 1 mutant; see description of *chy1* for phenotypes. (Zolman et al., 2000)
B29	Class 1 mutant; see description of *chy1* for phenotypes. (Zolman et al., 2000)
Peroxin 5 (pex5) Peroxisomal import receptor	*pex5* (B44) isolated as class 1 mutant, see description of *chy1* for phenotypes, and text for details of protein function. (Zolman et al., 2000)

Peroxisomal ABC transporter1 (pxa1) *Comatose (cts)* *Peroxisome defective 3 (ped3)* ATP Binding Cassette transporter with similarity to human X-linked adrenoleukodystrophy protein, acting in peroxisomal import of substrates	• *pxa1* (B40) isolated as a Class 1 mutant, see description of *chy1* for phenotypes. (Zolman et al., 2000) • *ped3* (LR43, LR81) mutants isolated by 2,4-DB resistance, 2,4-D sensitivity, and sucrose dependent seedling growth (cotyledon expansion). 2,4-DB resistance is dominant, while sucrose dependence is recessive. (Hayashi et al., 1998) • *cts* mutants were isolated because of reduced germination on media containing 3% sucrose after one month of after-ripening. *cts-1* and *cts-2* are recessive, embryonic mutations (not maternal effect). GA or short pre-chilling do not enhance germination, though extended prechilling increases germination to about 20%. Embryo, seedling and plant morphology is undistinguishable from wild-type. (Russell et al., 2000) • *pxa1* displays IBA resistant, IAA (and NAA d.n.s.) sensitive root elongation. *pxa1* have fewer lateral roots in the absence of auxins, and lateral root formation is non-responsive to IBA. IAA can induce lateral roots. *pxa1* seedlings germinate, but cotyledons do not expand in the absence of sucrose in either light or dark conditions. *pxa1* roots slightly shorter than WT. On soil, *pxa1* plants grow more slowly and are smaller. IBA resistance is dominant, while sucrose dependence is recessive. (Zolman et al., 2001b) • *cts* mutants can germinate on media with sucrose if testa and endosperm layers are removed. *cts* seedlings have enlarged lipid bodies in cotyledons, but peroxisomes are morphologically wild-type. Acyl-CoAs accumulate in *cts*, and levels of sucrose, glucose and fructose are modified. (Footitt et al., 2002)

(*pxa1/cts1/ped3* – continued)	• *ped3-1* to *–4* fail to establish in the absence of sucrose, but do not require sucrose after the expansion of green leaves. *ped1 ped3* double mutants display dwarf inflorescences, wavy leaves and are sterile. Increased lipid content of *ped3* seeds after 5 days of growth in light, compared to wild-type. Cell fractionation and immunolocalization studies show PED3 is localized to the glyoxysomal membrane. Glyoxysomes look morphologically normal in *ped3* mutants. (Hayashi et al., 2002)
Abnormal inflorescence meristem (aim1) Functions in ß-oxidation of fatty acids, similar to cucumber multifunctional protein with L-3-hydroxyacyl-CoA hydrolyase, L-3-hydroxyacyl-dehydrogenase, D-3-hydroxyacyl-CoA epimerase, and 3, 2-enoyl-CoA isomerase activities (TAIR database)	• *aim1* presents an abnormal inflorescence meristem with severely reduced fertility. Vegetative rosette phenotypes enhanced by short day conditions: leaves dark green and smaller, twisted. Shoot apices examined prior to transition to flowering look normal, but 5 days after transition, phenotypic differences are seen. Formation of abnormal or unrecognizable flower buds with some homeotic conversions of floral organs, inflorescence meristems terminate prematurely. Increased lateral branching. Small amount of seed produced after prolonged growth. Modified fatty acid composition. In the absence of sucrose, no germination of seeds produced from homozygote *aim1* mutant, but no reduction of germination of seeds from heterozygotes, suggesting maternal rescue. *aim1* roots resistant to a single concentration of 2,4-DB (2μM). (Richmond and Bleecker, 1999) • IBA resistant, with phenotypes like class 1 mutants (see *chy1*) - Zolman d.n.s.

Peroxisome defective 1 (*ped1-1*) Ketoacyl-CoA thiolase2 (*kat2*) Ketoacyl CoA thiolase; Predominantly expressed thiolase in early seedling growth, protein contains a peroxisomal targeting signal (PTS2).	• *ped1-1* (LR40) isolated as 2,4-DB resistant, 2,4-D sensitive mutant; displays sucrose dependent growth under constant illumination. When germinated with sucrose, can grow on soil and produce WT looking adult plant. Glyoxysomes 2-3-fold bigger than in WT. (Hayashi et al., 1998) • *ped1* is IBA resistant, with phenotypes like class 1 mutants (see *chy1*) (Zolman et al., 2000) • *kat2* is resistant to 2,4-DB and cotyledon expansion is sucrose dependent. The defect in lipid mobilization results in higher levels of triacylglycerol and total lipids in seedlings. Most cells contain much enlarged peroxisomes. (Germain et al., 2001)
<u>Putative IBA response or transport mutants:</u>	
Indole-3-butyric acid response 1 (ibr1)	Class 2 mutants: IBA resistant, IAA sensitive root elongation and lateral root formation; normal growth in the absence of sucrose; normal levels of the fatty acid eicosenoic acid; resistant to 2,4-DB, but not to 2,4-D or NPA. (Zolman et al., 2000)
Indole-3-butyric acid response 2 (ibr2)	Class 2 mutant (see *ibr1* for phenotype description). (Zolman et al., 2000)
Indole-3-butyric acid response 3 (ibr3)	Class 2 mutant (see *ibr1* for phenotype description). (Zolman et al., 2000)
Indole-3-butyric acid response 4 (ibr4)	Class 3 mutant: IBA resistant, IAA sensitive root elongation, but WT response to IBA for lateral root formation; Normal growth in the absence of sucrose; Normal levels of the fatty acid eicosenoic acid; Resistant to 2,4-DB, but not to 2,4-D or NPA. (Zolman et al., 2000)

Indole-3-butyric acid response 5 (ibr5)	Class 4 mutant: IBA resistant, IAA sensitive root elongation, but WT response to IBA for lateral root formation; Normal growth in the absence of sucrose; Normal levels of the fatty acid eicosenoic acid; Resistant to 2,4-DB, and also to 2,4-D and NPA (Zolman et al., 2000)
Indole-3-butyric acid response 6 (ibr6)	Class 4 mutant: see description of *ibr5* for phenotypic description. (Zolman et al., 2000)
Mutants initially identified as defective in light response	
Constitutively photomorphogenic 1 (cop1) *Embryo lethal 168 (emb168)* *Fusca 1 (fus1)* Protein contains a Zinc-binding domain and a domain homologous to the beta subunit of heterotrimeric G proteins. Interacts with CIP8, which acts as an ubiquitin E3 ligase	• Constitutive photomorphogenesis in dark, including short hypocotyls, open apical hook, leaf formation, expression of light regulated genes, chloroplast development, and anthocyanin accumulation. *cop1* does not affect phytochrome control of seed germination, and *cop1* plants do not show differences in expression of light-regulated genes in response to dark adaptation. (Deng et al., 1991) • Dark-grown *cop1* seedlings present cell differentiation characteristics similar to light-grown wild-type seedlings including irregular and enlarged epidermal cells and mature stomatal structures in cotyledons and hypocotyls. (Deng et al., 1992) • *cop1-6*, a week *cop1* mutant, severely inhibits lateral root formation, which is the antagonistic effect of the *hy5-1* mutation (which stimulates lateral root formation). *hy5-1 cop1-6* double mutants have increased number and length of lateral roots, indicating *hy5* can suppress the root defects of *cop1*. (Ang et al., 1998) • IBA resistant (d.n.s.); defective peroxisomes. (Hu et al., 2002)

De-etiolated 1 (det1) *Fusca 2 (fus2)*	• De-etiolated phenotype in the dark, including short hypocotyls, open apical hook, leaf formation, expression of light regulated genes, chloroplast development, and anthocyanin accumulation (50X higher than WT). Light grown plants are smaller and paler than wild-type plants grown in the same conditions. Seed germination is independent of light in *det1* mutants. (Chory et al., 1989) • *det1* roots contain large numbers of chloroplasts, while WT roots do not contain chloroplasts, but rather amyloplasts. (Chory and Peto, 1990) • IBA resistant (d.n.s.); *ted3* can suppress iba resistance of *det1-1*. (Hu et al., 2002)
<u>Mutants initially identified as defective in ethylene response</u>	
Ethylene insensitive 2 (ein2) *Cytokinin resistant 1(ckr1)* *Polar inhibitor resistant 2 (pir2)* *Enhanced response to ABA 3 (era3)* EIN2 proposed to act as bifunctional signal transducer; amino terminal end of protein has homology to Nramp metal ion transporters.	• IBA and 2,4-D resistant, IAA and NAA sensitive for root elongation. Resistant to auxin transport inhibitors. Lateral root formation sensitive to IBA. (Zolman et al., 2000) • All *ein2* alleles are completely insensitive to ethylene. They are also resistant to jasmonate and paraquat (oxygen radical releasing agent). *ein2* alleles also isolated as auxin transport inhibitor resistant, cytokinin resistant, delayed senescence and ABA hypersensitive mutants. (Alonso et al., 1999, Wang et al., 2002, and references therein; Ghassemian et al., 2000) • Root hairs shorter in *ein2-1* mutants, and root hair production and length can be reduced by treatment with auxin influx inhibitor CSI and 1-NOA. NAA can rescue root hair number and length in *ein2*. (Rahman et al., 2002)

Ethylene receptor 1 (etr1) *Ethylene insensitive 1 (ein1)* *(ein1)*	• Encodes a homodimeric membrane component. Binds ethylene using a copper cofactor. (Wang et al., 2002) • IBA, 2,4-D and auxin transport inhibitor resistant. (Zolman et al., 2000) • ETR1 initially isolated as a dominant ethylene insensitive plant. *etr1* loss-of-function alleles present a growth defect limiting plant size, and this was shown to be caused by enhanced sensitivity and exaggerated response to ethylene (Cancel and Larsen, 2002)

[1] Mutations with different names but affecting the same gene are grouped together in the same row.

[2] In most cases, results presented in different publications are placed in different paragraphs.

Also IBA resistant (see Table 1.3 for phenotypes): *axr1, axr2, axr3, aux1, shy2, IAA28* (our d.n.s. and Zolman et al., 2000)

Table 1.2: Phenotypes of Arabidopsis mutants defective in auxin transport.

Full gene name, (symbol) ***Aliases (Allelic gene names)*** **Proposed protein function**	**Mutant phenotypes**
Putative auxin influx mutants	
Auxin1 (aux1) *Polar inhibitor resistant1 (pir1)* *ACC resistant (accr)* *Wavy root5 (wav5)* auxin influx carrier (similar to amino-acid permeases) (Bennett et al., 1996)	• *aux1* root elongation is resistant to inhibition by IAA, IBA, 2,4-D, ACC, ethylene, BA. Roots are agravitropic and elongate faster in some conditions. Hypocotyls shorter in the dark, and reorient faster in response to gravistimulation. Decreased lateral root formation that is resistant to IAA and 2,4-D. (Maher and Martindale, 1980; Mirza et al., 1984; Pickett et al., 1990; Timpte et al., 1995) • *aux* is not NAA resistant, but NAA can rescue agravitropic nature of root growth. (Yamamoto and Yamamoto, 1998) • *aux1-7* is IAA and IBA resistant for inhibition of primary root elongation, but not for lateral root formation. (Zolman et al., 2000) • *aux1-7* has less saturable 2,4-D uptake, NAA restores gravitropic response of *aux1-7* ; 2,4-D partially rescues gravitropic defect; NPA blocks NAA rescue of gravitropic response. (Marchant et al., 1999) • *aux1-7* forms 50% less lateral root than WT at 5-13 dag, NAA can rescue the lateral root phenotype. Increased accumulation of IAA in leaves, and modifed accumulation in roots. (Marchant et al., 2002)

Auxin resistant4 (axr4) *Reduced root gravitropism1 (rgr1)* Mutant phenotypes suggest defect in auxin influx carrier	• *axr4* roots are IAA and 2,4-D resistant (5 fold for both), ABA resistant at 10^{-5} M only, not resistant to ACC or kinetin. Reduced root gravitropism, root slightly longer, reduced number of lateral roots. (Hobbie and Estelle, 1995) • *rgr1* roots show reduced gravitropism, clockwise curling instead of wavy growth, reduced lateral root formation, shorter primary root in light at 12 dag and more, IAA (6-fold), 2,4-D (3.3-fold), NPA (5.4-fold), resistant, less resistant to TIBA (1.7-fold) NAA (1.8-fold). Resistant to 2,4-D for hypocotyl elongation (more in light than dark). Not ABA, ethylene or cytokinin resistant. (Simmons et al., 1995a) • *axr4* not NAA resistant, but resistant to IAA and 2,4-D. NAA can restore gravitropism of *axr4* roots. In contrast, *axr1* resistant to all three auxins and gravitropism was only slightly affected by auxins in medium. These data suggest *axr4* is defective in an auxin influx carrier. (Yamamoto and Yamamoto, 1999)
<u>Putative auxin efflux carriers</u>	
Pin-formed1 (pin1) putative auxin efflux transporter	• Few flowers formed – inflorescence ends in pin-like structure instead, few abnormal flowers: large petals, no stamens or ovary; reduced IAA transport and IAA levels. (Okada et al., 1991) • Drastically fasciated stems formed, seedlings with three cotyledons produced, increased vascular tissue development. (Galweiler et al., 1998) • Organ initiation affected in *pin1*, but not meristem organization; organ separation is defective. (Vernoux et al., 2000)

Agravitropic1 (agr1) *Ethylene insensitive root1 (eir1)* *Pin-formed2 (pin2)* *Wavy root6 (wav6)* Putative auxin efflux transporter	• *agr1* has agravitropic roots (emergence and reorientation). (Bell and Maher, 1990) • All *agr1* alleles tested have roots with increased sensitivity to NAA at a single conc. (0.3μM), *agr1* shows resistance to ACC and TIBA, reduced efflux of preloaded H3-IAA. (Chen et al., 1998) • *agr1* defective in root turning assay - can't redistribute IAA or NAA applied to one side of root. (Utsuno et al., 1998) • *eir1* seedlings have agravitropic roots that are ethylene resistant. Resistant to inhibition of root elongation by endogenous auxin increase caused by *alf1-1* but not to ectopic root formation by *alf1-1*, not resistant to inhibition of root elongation by exogenous auxin, resistant to TIBA. Longer primary root, less wavy root growth. (Luschnig et al., 1998) • *pin2* roots agravitropic and short, more sensitive to NAA and IAA, but no difference in 2,4-D response. (Müller et al., 1998)
pin-formed3 (pin3) Putative auxin efflux transporter	Hypocotyls and roots shorter in light, but not in dark, higher percentage of hookless hypocotyls in dark, reduced hypocotyl photo- and gravitopism. (Friml et al., 2002b)
Pin-formed4 (pin4) Putative auxin efflux transporter	Abnormal root meristem, abnormal embryo patterning, higher IAA levels in roots. (Friml et al., 2002a)

A. thaliana multidrug resistance like1 (Atmdr1) ATP binding cassette containing transporter (putative auxin efflux carrier)	• Epinastic cotyledons and first leaves (can be phenocopied by 2,4-D). Epinasty more visible under dim light than under high light. Rosette leaves curled and crinkled, bolt has reduced growth rate, though eventually same length as WT. Some etiolated hypocotyls are wavy. (Noh et al., 2001) • Reduced IAA transport in hypocotyls and inflorescence stems. MDR1 expressed in yeast cells binds NPA. (Reviewed in Luschnig, 2002)
A. thaliana P glycoprotein 1 (Atpgp1) ATP binding cassette containing transporter (putative auxin efflux carrier)	• Transgenic overexpressors (OE) and antisense (AS) plants produced. OE have longer hypocotyls, and AS have shorter hypocotyls in the light (white, red or blue). Difference between WT and AS or OE seedlings maximal at about 10μmoles m-2 s-1, no difference at 1 or 100 μmoles m-1 s-1. Etiolated hypocotyls no diff. from WT. (Sidler et al., 1998) • *pgp1* hypocotyls same length as WT in dark and low white light (2μmoles m^{-2} s^{-1}). IAA transport unchanged in *pgp1* (Noh et al., 2001) • Cloning in Dudler and Hertig (1992).
pgp1-1 mdr1-1 double mutant	• Hypocotyls short and wavy in the dark, short hypocotyl in light too, very epinastic cotyledons and leaves, leaves also crinkled, irregularly shaped and severely curled. Plants stunted with loss of apical dominance. Filaments of anthers short resulting in reduced fertility. Reduced IAA transport in hypocotyls and inflorescence stems. (more than in *mdr1* single mutants). (Noh et al., 2001)

Putative auxin transport regulators	
Interfascicular fiberless (ifl1) *Revolute leaf (rev)* homeodomain Leucine zipper protein (HD-ZIP)	No interfascicular fiber, secondary xylem abnormal, long stems, dark green leaves with delayed senescence, reduced number cauline branches, secondary rosette formation, short primary root, delay in lateral root formation, formation of pin-like inflorescences in null allele *ifl1-2*. Reduced IAA transport in stems, reduced efflux from hypocotyls, reduced NPA binding. (Zhong and Ye, 2001)
Lopped1 (lop1) Unknown identity, possibly involved in transport regulation	Disoriented, rotated or split midveins, lateral roots abnormally curved, short primary root. Male sterile, reduced IAA basipetal transport in stems. (Carland and McHale, 1996)
Pinoid1 (pid1) Ser/Thr protein kinase (Cloning in Christensen et al., 2000)	• Flowers with more petals, seedlings with three cotyledons (85%), reduced auxin transport when stems have stopped elongating. (Bennett et al., 1995) • Abnormal petal and carpel venation pattern. PID overexpressors: 10% extreme dwarves, sterile=homozygous for transgene, others loss of apical dominance, short, no lateral roots, agravitropic, small crinkled leaves, short primary root, NAA resistant growth (if considered as percent of no hormone). (Christensen et al., 2000) • Strong En alleles: pin-like inflorescences, few abnormal flowers w/ few or no sepals or stamens, more petals, trumpet shaped pistil that produces almost no seeds, or only carpelloid structures. Overexpression lines: loss of hypocotyl and root gravitropism, reduced root and hypocotyl

pid1 – continued	elongation, delay in lateral root formation and collapse of primary root meristem, reduced apical dominance in inflorescence stem (NPA can rescue these phenotypes) Increased PID expression in aerial parts results in more lateral root formation. (Benjamins et al., 2001)
Root curl in NPA1 (rcn1) PP2A regulatory subunit A	Root curl in NPA, auxin efflux less sensitive to inhibition by NPA. (Garbers et al., 1996) Lateral root growth shows reduced NPA sensitivity. *rcn1* has increased root basipetal transport of IAA, and reduced sensitivity of root acropetal transport to NPA. (Rashotte et al., 2001)
Transport inhibitor response1 (tir1)	See auxin response mutants (Table 1.3)
Transport inhibitor response2 (tir2)	NPA resistant; short hypocotyl and reduced number of lateral roots on control media. (Jensen et al. 1997 – International meeting on Arabidopsis research meeting abstract) From TAIR: Defective in a variety of auxin-regulated growth processes including hypocotyl elongation and lateral root formation.
Transport inhibitor response3 (tir3) *Dark Overexpressor of CAB1 (doc1)* *BIG* Calossin-like (possibly calmodulin binding?)	• Deficient in lateral root formation, reduced apical dominance, short siliques, pedicels, roots and inflorescence stem. Reduced NPA binding and IAA transport in inflorescence stems. (Ruegger et al., 1997) • *doc* mutants overexpress a subset of light induced genes in the dark. Some of these defects are suppressed by the auxin overproducing *yucca* mutation, suggesting a defect in auxin transport. (Gil et al., 2001)

Transparent testa4 (*tt4*) Chalcone synthase Involved in biosynthesis of flavonoids, putative endogenous transport inhibitors	Reduced apical dominance, shorter primary inflorescence, more lateral and adventitious roots, elevated IAA transport in inflorescence stems and hypocotyls. (Brown et al., 2001)

Table 1.3: Phenotypes of IAA resistant mutants involved in auxin response and of related mutants

Mutant / gene name (symbol) ***Aliases (allelic gene names)*** **Proposed protein function**	**Mutant phenotypes**
Proteins involved in regulated proteolysis through the ubiquitin/proteasome pathway	
Auxin resistant 1 (axr1) Similarity to NH-terminal half of E1 ubiquitin activating protein *(AXR1* cloning in Leyser et al., 1993) Nuclear protein thought to cause activation of RUB (related to ubiquitin) protein in nucleus (as a dimer with ECR1). (del Pozo et al., 1998)	• 2,4-D resistant (50-fold) and IAA resistant (8-fold). Small crinkled rosette leaves with short petioles, reduced apical dominance, small flowers, poor male fertility, short stamens, no change in 2,4-D metabolism. (Estelle and Somerville, 1987) • Characterization of *axr1-3* (weak allele), and *axr1-12* (strong allele). *axr1-12* adult plant half WT height, apical dominance reduced, short hypocotyls in the dark, but not in the light. Reduced root gravitropic response, primary root slightly shorter, resistant to 2,4-D inhibition of rosette leaf weight. Cell length in stems is normal, so reduction in stature probably due to a reduction of cell number. (Lincoln et al., 1990) • *axr1-3* and *axr1-12* roots are BA and ethylene (slightly) resistant, and have a reduced number of lateral roots. *aux1 axr1* double mutants show additivity of phenotypes (Timpte et al., 1995) • *axr1-3* contains 2-3-fold more IAA than wild-type controls, yet it can suppress phenotypes produced by transgene-mediated auxin overproduction (Romano et al., 1995) • *axr1-12* roots elongate faster, root epidermal cells longer, reduced number of root hairs (50%), stem epidermal cells shorter. (Cernac et al., 1997) • *axr1* has a higher percentage of elongating inflorescences than wild-type, but the same number of

axr1 – continued	inflorescence meristems. Apically applied NAA inhibits lateral shoot elongation in WT, but not in *axr1*. (Stirnberg et al., 1999) • Reduced hypocotyl gravitropism and phototropism, and reduced response to unilateral application of auxin. 2,4-D resistant hypocotyl growth (Watahiki et al., 1999) • Low concentration IAA (0.1μM) can stimulate hypocotyl elongation in *axr1-12*, but the mutant is resistant to inhibition by higher concentration. Number of cells in epiderminal cell file of *axr1-12* hypocotyls is the same as wild-type, so modifications in hypocotyl length probably due to change in elongation rather than cell division. (Collett et al., 2000) • Characterization of the *axr1-24* allele, isolated by resistance to methyl jasmonate (MeJa). IAA resistance level similar to *axr1-3*. Short plant, reduced apical dominance, number of siliques, length of siliques, and male fertility, like other *axr1* mutants. Etiolated hypocotyls not shorter than wild-type (unlike *axr1-3* and *axr1-12*). Root growth resistant to MeJa, ACC, epibrassinolide and germination partly resistant to ABA. Resistant to salicylic, ferulic and gallic acids, and increased susceptibility to the fungus *Pythium irregulare*. (Tiryaki and Staswick, 2002) • *axr1-3* root elongation and induction of VSP transcript is MeJa resistant. *axr1-3* shows delay in cold induced COR gene induction. *axr1* show a synergistic effect with the putative E2 mutant *cop10-4* for deetiolation in the dark (as determined by short hypocotyl and induction of light regulated genes), but additive effects for 2,4-D resistance in the double mutants. (Schwechheimer et al., 2002)

ECR1 Nuclear protein thought to act with AXR1 to activate RUB protein in nucleus. (del Pozo et al., 1998)	Phenotype of dominant negative form of *ECR1*: Rosette, leaves, inflorescences smaller/shorter, resulting in a short bushy plant (similar to *axr1* phenotypes). Root system similar to wild-type. Reduction in seed production because of shorter stamens. Root growth is not auxin resistant (d.n.s.), but deficient in auxin-induced expresssion of the *IAA2* gene. Reduced RUB modification of CUL1. (del Pozo et al., 2002)
RUB conjugating enzyme 1 (rce1) Works together with AXR1-ECR1 to promote formation of stable conjugate of RUB with AtCUL1. (del Pozo and Estelle, 1999)	Defective root gravitropism, reduced organ length throughout development: small leaves, short petioles, round and crinkled leaf blades. Short inflorescence and increase lateral branching, similar to *axr1*. Roots are 2,4-D resistant for elongation and lateral root formation. Reduced IAA induction of IAA2 and BA3::GUS genes, and increased stability of IAA7. Reduction in jasmonic acid response for root growth inhibition. Double mutant with *axr1-12* is dosage sensitive (though both mutations are recessive): homozygous *axr1-12* and heterozygous *rce1* have seedling lethal phenotype. Homozygous *axr1-12 rce1* double mutants: loss of hypocotyl and roots, seedlings are cotyledons only, lethal. Vascular development also severely reduced in double mutant cotyledons. (Dharmasiri et al., 2003)
auxin resistant 6 (axr6) *Cullin1 (cul1)* Encodes CUL1, Component of the SCF^{TIR1} complex (Hellmann et al., 2003)	Homozygous *axr6* have no root, high frequency of single cotyledons, reduced vascular development in cotyledons. *axr6* heterozygotes : small plants with crinkled leaves, siliques more vertical, loss of apical dominance, reduced number of siliques. Heterozygotes are 2,4-D, NAA, BA and kinetin resistant in root elongation assays, and are resistant to IAA in hypocotyl elongation assays, and have defects in root gravitropism. *axr6* hets also have higher expression levels of the auxin responsive BA3::GUS reporter gene. (Hobbie et al., 2000)

Arabidopsis SKP1-like (ask1) Component of the SCF^{TIR1} complex	2,4-D resistant root growth. Reduced number of lateral roots. Incomplete penetrance of *ask1-1* mutation for 2,4-D resistance. (Gray et al., 1999)
Transport inhibitor response 1 (tir1) *Weak ethylene insensitive 1 (wei1)* F-box and LRR containing protein. Component of the SCF^{TIR1} complex (see text for details)	• Isolated as NPA resistant, but no defect in IAA transport in inflorescence stems. *tir1* is more resistant to 2,4-D than IAA, and shows a synergistic effect with *axr1* for 2,4-D resistance. (Ruegger et al., 1998) • Reduced number of lateral roots in the presence and absence of 2,4-D. TIR1 overexpression in the light results in lateral root development and inhibits primary root elongation, and in the dark, inhibits hypocotyl elongation and promotes apical hook opening. These phenotypes are similar to those caused by auxin application (IAA or 2,4-D) (Gray et al., 1999) • *wei* is a new allele isolated as a weak ethylene insensitive mutant. 2,4-D resistant root and hypocotyl elongation. (Alonso et al., 2003)
RBX1 RING-H2 finger protein. Component of the SCF^{TIR1} complex	• RBX antisense (AS) plants: most die as young seedlings, with purple cotyledons and long hypocotyls. Surviving plants are small, dark green, loss of apical dominance, form clusters of flowers. These phenotypes are not recovered in the next (T2) generation. (Gray et al., 2002) • RBX AS plants (phenotypes of weaker suppression): loss of apical dominance and organ identity, AS line not 2,4-D resistant. Reduced elongation of root hairs and number of lateral roots. Root growth methyl jasmonate (MeJa) resistant. Delayed induction of COR genes in response to cold treatment. (Schwechheimer et al., 2002) • RBX sense, cossuppressed line (strong phenotype): small plants with a single inflorescence stem.

RBX1 – continued RBX1 interacts directly with RCE1 and is therefore suggested to act as E3 for RUB1 modification of CUL1 (Dharmasiri et al., 2003)	Root growth 2,4-D resistant. Reduced number of lateral roots. Root growth and VSP transcript induction are MeJa resistant. (Schwechheimer et al., 2002) • RBX overexpressing (OE) lines (35S::RBX1): dark grown seedlings are partially de-etiolated, with shorter hypocotyl and no apical hook. Light grown seedlings have smaller cotyledons, fewer lateral roots, small misshapen rosette leaves. Plants short and bushy, with small flowers. OE lines are also 2,4-D resistant in root elongation assays. (Gray et al., 2002)
<u>Aux/IAA proteins</u>	
Suppresor of hy and short hypocotyl 2 (shy2) IAA3	• *shy2* isolated independently as a suppressor of *phyB* and *hy2* mutations. *shy2-2* and *shy2-3* are semi-dominant mutations. Leaves curl upwards. Hypocotyls short and no apical hook in dark with 2% sucrose. True leaves produced in dark after 23 days. *shy2-2* specifically affected in response to red light, not to blue or far-red light. Root length also affected: *shy2-2* root longer and *shy2-3* root shorter than WT. (Reed et al., 1998) • *shy2-2* and *shy2-3* have large cotyledons that curl up, with short hypocotyls, and adult plants are extreme dwarves with curled up leaves. *shy2-2* is semidominant for adult phenotypes. *shy2-22* and *shy2-24* mutants are loss-of-function (l-o-f); hypocotyls grow slightly faster but are the same final length as wild-type, *shy2-22* has slightly larger cotelydons, but they are not curled up and adult l-o-f mutants look normal. *shy2-22* and *-24* hypocotyls slightly shorter in the dark with sucrose after 6 days. Both l-o-f and gain-of-function (g-o-f) *shy2* mutations result in short root in the light. L-o-f mutations cause an increased number of lateral roots and g-o-f mutations repress lateral and adventitious root formation. Gravity response: *shy2-24* roots had a faster than WT

shy2 / IAA3 - continued	response to a change in gravity response, *shy2-2* and *shy2-22* can respond to gravity, though slower than WT. *shy2*-2 is IAA (2-fold), ABA, BA, NPA and ACC resistant. *shy2-22* and *shy2-24* root elongation stimulated by low concentrations of IAA, BA, NPA, but not ABA. (Tian and Reed, 1999)
Auxin resistant 2 (axr2) IAA7	• *axr2-1* is a dominant mutation (2,4-D resistance). *axr2* mutants are very small, dark green, with normal fertility. Small leaves with short petioles, inflorescences have defect in negative gravitropism. Roots have a slow growth rate, no root hairs, and show reduced gravitropism. More resistant to IAA (65 fold more than WT) than to 2,4-D (10 fold more than WT). Resistant to ethylene and ABA (Wilson et al., 1990) • Analysis of 7 day-old *axr2* mutants: in light and dark, *axr2* hypocotyls shorter, roots longer, though effect on hypocotyls more dramatic in the dark. Reduced gravity response of hypocotyls and roots seen for *axr2*. *axr2* cells shorter in seedlings and adult plants. In inflorescence stems, reduced cell length is accompanied by a 4-fold increase in stomata density. (Timpte et al., 1992) • *axr2* more resistant than wild-type to inhibition of root elongation by IAA, but more sensitive to induction of lateral root primordia by 2,4-D and IAA. (Boerjan et al., 1995) • Low concentration IAA stimulates root elongation in *axr2* mutants, more resistant than *axr1* to root elongation inhibition by higher conc. of IAA; *axr2* has more lateral roots than WT in the dark and root production can be stimulated by exogenous IAA. *axr2* has short hypocotyl in the dark and IAA has a very small effect on hypocotyl elongation, leading to the suggestion that AXR2 is more important for IAA response in the hypocotyl than in the root. (Knee and Hangarter, 1996) • Analysis of *axr2-5* null mutant: very similar to wild-type for root length, root hairs, gravitropism;

axr2/IAA7 continued	the only statistically significant difference is a slightly longer hypocotyl in the light, especially evident at day 5 and 6 after germination. *axr2-5* has normal root auxin response and lateral root formation. • *axr2-1* hypocotyls shorter in dark and in red, far-red and blue light. Dark grown *axr2-1* lack apical hook and produce true leaves and occasionally even floral buds. (Nagpal et al., 2000) *axr2-1* has normal lateral root formation after 8 days without auxin or 4 days of IAA treatment, but IBA induces more than twice as many lateral roots as in WT (Rogg et al., 2001)
Bodenlos 1 (bdl1) (German for "bottomless") IAA12	• Strong phenotype results in absence of root (basal peg instead) and hypocotyl in seedlings, with cotyledons hyponastic. Weaker phenotype is absence of primary root only. Adult *bdl* mutants are dwarf, have rolled-up leaves, reduced apical dominance (bushy), defective vasculature, fertile with normal looking flowers. Resistant to 2,4-D mediated inhibition of root and hypocotyl elongation. Embryo development is abnormal starting at the 2-cell stage. Abnormal starch accumulation in apical meristem revealed by lugol staining. No starch in basal peg. (Hamann et al., 1999) • Cloning of *BDL*. BDL/IAA12 and MP/ARF5 interact in yeast two-hybrid assays, and are co-expressed in some cells. (Hamann et al., 2002)
Solitary root 1 (slr1) IAA14	No lateral roots formed on light-grown, 14 day old seedlings. Almost no lateral roots formed following IAA, NAA or 2,4-D treatment. Rare lateral roots formed on 1mM 2,4-D (very high conc.). Very few root hairs, mainly at root/shoot junction. Reduced gravity response of roots and hypocotyls. Altered cell elongation profile in hypocotyls (d.n.s.). Root elongation assays: resistant to IAA, 2,4-D, NAA, TIBA and PCIB, slightly more sensitive to ABA than WT; WT response to

slr1 / IAA14 continued	BA, ACC, NPA and HFCA. Normal formation of apical hook in dark. Shorter inflorescence axis, increased apical dominance and more slender stems. Reduced expression of BA::GUS auxin responsive construct in roots. (Fukaki et al., 2002)
Auxin resistant 3 (axr3) IAA17 Cloning of *AXR3* in Rouse et al., 1998	• *axr3-1* plants short, reduced number of inflorescence and shoot branching, short hypocotyl in dark but not in light. Reduced root length, increased number of adventitious roots, no root hairs, many lateral root initials on 1 week-old *axr3* seedling. Root growth IAA (500-fold), ACC and BA resistant. Differences in hypocotyl elongation in dark between *axr3* and WT can be rescued by cytokinin (BA). SAUR-AC gene expression in *axr3* background is reminescent of expression in WT treated with auxin. *axr3* explants produce more roots in the presence or absence of 2,4-D. Semi-dominant mutation. (Leyser et al., 1996) • *axr3-1* hypocotyls shorter in dark and in red, far-red and blue light. Dark grown *axr3-1* lack apical hook and produce true leaves (like *axr2-1*). *axr3-1* hypocotyls longer than wild-type in far-red light. (Nagpal et al., 2000) • *axr3-1* in white light: hypocotyls initially grow faster than WT Col-0 and are longer at days 2-3, but stop growing around day 4, so are shorter than WT at day 5. *axr3-1* less sensitive to hypocotyl elongation inhibition by IAA (Collett et al., 2000) • *axr3-1* has more lateral roots than WT in the absence of auxin, less in the presence of IAA and approx. equal numbers in the presence of IBA (no statistical analysis done) (Rogg et al., 2001)

iaa28 IAA28	Resistant to IAA, ACC and BA in root elongation assays, but normal response to ABA, MeJa, epibrassinolide; normal ACC response in hypocotyls. Lateral root numbers drastically reduced, while root and hypocotyl length are normal and respond "almost normally" to high temperatures. Exogenous IAA and IBA can induce lateral root formation in *iaa28-1*, though not as many as in WT. *iaa28-1* seedlings have fewer root hairs, plants are shorter, with more stems and lower seed yield (decreased size and number of siliques), primary root less branched. Aerial phenotypes are recessive, while root phenotypes are semi-dominant. (Rogg et al., 2001)
<u>Auxin response factors</u>	
Ettin (ett) Auxin response factor ARF3 ETTIN lacks domains III or IV proposed to be important for dimerization with other ARFs and Aux/IAA proteins. Therefore could act by competing with other ARFs for binding to AREs in auxin-responsive promoters (Nemhauser et al., 1998).	• Defects in gynoecium (female reproductive structure) development, with alterations due to merging of apical and basal regions and development of abaxial into adaxial tissues. Gynoecia display: split styles and stigma, decrease in ovary size, reduced septum formation, etc.. Approximately 50% of flowers produce additional sepals and petals and/or some anther loss (Sessions and Zambryski, 1995). • Cloning of *ETTIN*: Amino-terminus of the protein contains a DNA binding domain highly similar to that of ARF1, MP and other ARFs. C-terminal half is unique and is of unknown function (Sessions et al., 1997). • Weak *ett* alleles are more sensitive to disruption by the polar auxin transport inhibitor NPA (Nemhauser et al., 2000).

Monopteros (mp) ARF5 Cloning of *MP*: expression studies show protein is nuclear. (Hardtke and Berleth, 1998)	• *mp* seedlings lack hypocotyl, radicle and root meristem, defect that is seen in octant stage embryo. Variable position and number of cotyledons (often only one or two fused), which are hyponastic. Roots can be produced in tissue culture. Shoot meristem normal. Reduced vascular development in *mp* cotyledons. (Berleth and Jürgens, 1993)
Massugu1 (msg1) (Japanese for "straight") *Non-phototropic hypocotyls 4 (nph4)* *Transport inhibitor response 5 (tir5)* ARF7	• *msg1* : No curvature induced by unilateral application of IAA on hypocotyls. Leaves of different alleles either epi- or hyponastic. Roots shorter than wild-type in light. Hypocotyl elongation is 2,4-D resistant, but root elongation is as sensitive as WT to 2,4-D. *msg1* hypocotyls not ACC resistant. *msg1* leaves resistant to 2,4-D induced chlorosis. Reduced hypocotyl gravitropism. (Watahiki and Yamamoto, 1997) • *msg1* hypocotyls have reduced gravitropism and phototropism, and reduced response to unilateral application of auxin. 2,4-D resistant hypocotyl growth. (Watahiki et al., 1999) • *nph4* seedlings have normal hypocotyl length, but faster apical hook opening in the dark. Hypocotyl length similar to WT in blue and red light. Light-grown adult plants have epinastic or hyponastic rosette leaves. Reduced hypocotyl phototropism and absent red light induced hypocotyl curvature. Hypocotyl elongation is IAA, 2,4-D and NAA resistant compared to WT. (Stowe-Evans et al., 1998) • *nph4* null mutant phenotypes are suppressed by ethylene application. Ethylene suppression does not occur in the presence of NPA, indicating auxin transport is required for ethylene dependent suppression of *nph4* mutant phenotypes (Harper et al., 2000)

Chapter 2: Characterization of the *rib1* mutant of Arabidopsis

The *rib1* mutant is resistant to indole-3-butyric acid, an endogenous auxin in *Arabidopsis thaliana*

(Poupart, J, Waddell, CS (2000) Plant Physiol 124: 1739-1751)

Title of article:

The *rib1* mutant is resistant to indole-3-butyric acid, an endogenous auxin in *Arabidopsis thaliana*

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Footnotes:

This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada to C.S.W. J.P. was funded by post-graduate scholarships from the Fonds pour la formation des chercheurs et l'aide à la recherche and the Natural Sciences and Engineering Research Council of Canada.

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Abbreviations used: IBA: Indole-3-Butyric Acid; IAA: Indole-3-Acetic Acid; 2,4-D: 2,4-Dichlorophenoxyacetic Acid; NAA: Napthalene Acetic Acid; NPA: Naphthylphthalamic Acid; TIBA: 2,3,5-Triiodobenzoic Acid; HFCA: 9-Hydroxyfluorene-9-Carboxylic Acid; No-0: Wild-type Nossen ecotype of *Arabidopsis thaliana*; SSLP: Simple Sequence Length Polymorphism; CAPS: Cleaved Amplified Polymorphic Sequences.

Abstract

The presence of indole-3-butyric acid (IBA) as an endogenous auxin in Arabidopsis has been recently demonstrated. However, the *in vivo* role of IBA remains to be elucidated. We present the characterization of a semi-dominant mutant that is affected in its response to IBA, but shows a wild-type response to indole-3-acetic acid (IAA), the predominant and most studied form of auxin. We have named this mutant *rib1* for *resistant to IBA*. Root elongation assays show that *rib1* is specifically resistant to IBA, to the synthetic auxin 2,4-dichlorophenoxyacetic acid, and to auxin transport inhibitors. *rib1* does not display increased resistance to IAA, to the synthetic auxin naphthalene acetic acid, or to other classes of plant hormones. *rib1* individuals also have other root specific phenotypes including a shortened primary root, an increased number of lateral roots and a more variable response than wild type to a change in gravitational vector. Adult *rib1* plants are morphologically indistinguishable from wild-type plants. These phenotypes suggest that *rib1* alters IBA activity in the root, thereby affecting root development and response to environmental stimuli. We propose models in which RIB1 has a function in either IBA transport or response. Our experiments also suggest that IBA does not use the same mechanism to exit cells as does IAA and we propose a model for IBA transport.

Introduction

Auxins are an important class of plant hormones that have been implicated in all aspects of plant growth and development. Numerous physiological and genetic studies have shown auxins to be involved in phenomena as diverse as tropisms, cell enlargement and division, lateral branching of shoots and roots, vascular differentiation, and early embryonic development (Davies, 1995; Hobbie, 1998). Although indole-3-acetic acid (IAA) is the most studied form of auxin, other auxins are also present in plants. Indole-3-butyric acid (IBA) is a naturally occurring auxin identified in several plant species including *Arabidopsis thaliana* (Epstein and Ludwig-Müller, 1993; Ludwig-Müller et al., 1993). In Arabidopsis and other plants, the level of free IBA is comparable to that of free IAA, suggesting that IBA is physiologically relevant. For example, in both tobacco leaves and Arabidopsis seedlings, levels of free IBA represent approximately 25 to 30% of the total free auxins (Sutter and Cohen, 1992; Ludwig-Müller et al., 1993). In elongating pea internodes free IBA represents about half of the free auxins (Nordström et al., 1991). Although IBA represents a significant proportion of the free auxin pool, its total amount, which includes both free and conjugated forms, is generally much lower than that of IAA. In Arabidopsis, the amount of conjugated IAA is approximately eight times higher than the level of conjugated IBA. The mode of conjugation also differs between the two auxins; in Arabidopsis, the majority of IAA conjugates are linked to amino acids, while most of the IBA conjugates are linked to sugars (Ludwig-Müller et al., 1993).

The occurrence of IBA as a natural constituent of plants was recognized as early as 1954 (Blommaert, 1954), but its physiological role is still unknown. Early studies that examined the effects of exogenous auxin application found IBA to be more effective than IAA in promoting the formation of adventitious roots (Zimmerman and Wilcoxon, 1935). Since that time, IBA has become the preferred auxin to induce root formation on cuttings and in tissue culture (Hartmann et al., 1997). This fact may reflect an *in vivo* role of IBA in root formation. IBA could act directly as a distinct auxin or indirectly through conversion to IAA. Interconversion of IBA and IAA has been demonstrated to occur in

maize, Arabidopsis and several other plant species (Epstein and Ludwig-Müller, 1993). There is no experimental evidence, however, that shows that conversion of IBA to IAA is necessary for IBA action. IBA treatment of peas causes an increase in endogenous levels of both IBA and IAA, but only IBA levels increase and remain high in the tissue that form roots (Nordström et al., 1991). This result suggests that IBA is in fact the active auxin in production of roots, since its presence, but not that of IAA, is correlated with root initiation. Similar results are obtained in the woody species *Populus tremula*; no evidence of IBA conversion to IAA is found when radiolabelled IBA is applied to cuttings to induce adventitious root formation (Pythoud and Buchala, 1989). In a recent report, Yang and Davies (1999) demonstrate that IBA can also promote stem elongation in peas, and they postulate that IBA is a physiologically active form of auxin in stem elongation in intact plants.

Characterization of mutants in IAA response and of IAA induced genes over the last 10 to 15 years has contributed extensively to our understanding of IAA transport, signal transduction and the role of this auxin in plant development. One family of auxin inducible genes, *Aux/IAA,* contains at least 25 members in Arabidopsis, and these genes encode short-lived nuclear proteins that have been proposed to be transcriptional regulators of downstream genes responsible for mediating auxin-regulated processes (Abel et al., 1994; Kim et al., 1997). A number of IAA resistant mutants have been isolated in Arabidopsis; they have confirmed the importance of IAA in embryonic development, root formation, cell elongation, gravity response, and apical dominance (Hobbie, 1998; Hobbie et al., 2000). In a few cases, the affected genes have been cloned; these examples have shown that auxin resistance can occur as a result of defects in auxin signal transduction or auxin transport (Rouse et al., 1998; Bennett et al., 1996).

IAA undergoes active polar transport from its point of synthesis in the apex of the shoot to its points of action. Transport occurs in a cell to cell fashion that is mediated by both influx and efflux carriers. Polarity of transport is achieved through localization of the efflux carrier to the basal side of cells (Lomax et al., 1995). Molecular and genetic studies in Arabidopsis have recently confirmed the existence of specific auxin carriers that had been suggested by earlier physiological studies. A single putative influx carrier has been identified; it is encoded by the *AUX1* gene and is expressed in root tips (Bennett

et al., 1996). AUX1 has homology to amino acid transporters suggesting that it facilitates the uptake of IAA, an amino acid-like molecule. A component of a specific IAA efflux carrier is encoded by the *AGR1/EIR1/PIN2/WAV6* gene and has homology to bacterial transmembrane transporters (Chen et al., 1998; Luschnig et al., 1998; Müller et al., 1998; Utsuno et al., 1998). Protein localization studies have shown that AGR1 functions in a well-defined subset of root epidermal and cortical cells (Müller et al., 1998). Multiple homologs of *AGR1* have been found in the Arabidopsis genome, providing evidence for the existence of a family of efflux carriers. Together, these findings suggest that each member may be specific for a certain tissue, developmental stage or environmental response. For example, the *PIN1* gene is one member of this family that has been implicated in auxin transport in stems and in floral development (Okada et al., 1991; Gälweiler et al., 1998).

In contrast with IAA, IBA has been largely ignored in genetic and molecular studies. Limited physiological experiments have been reported in the literature. From these studies, we know that IBA, like IAA, undergoes polar transport (Went and White, 1938), and that auxin transport inhibitors can inhibit this transport (Leopold and Lam, 1961). IAA can compete with IBA uptake, suggesting that IAA and IBA most likely utilize the same influx carrier (Ludwig-Müller et al., 1995c). To the best of our knowledge, no study has directly addressed the subject of IBA efflux. Conflicting results about the rate of transport of IBA appear in the literature: some report that IBA and 2,4-D (2,4-dichlorophenoxyacetic acid, a synthetic auxin) are transported more slowly than IAA, while others conclude that both IBA and IAA are transported at the same rate (Epstein and Ludwig-Müller, 1993; Ludwig-Müller et al., 1995c). These discrepancies can be explained by referring to differences in plant species studied, plant organs used and experimental design.

We have isolated an Arabidopsis mutant that is specifically resistant to the auxins IBA and 2,4-D, but not to the auxins IAA or NAA (naphthalene acetic acid, a synthetic auxin). This paper reports the first phenotypic characterization of a mutant that can discriminate between the two endogenous auxins of Arabidopsis, IAA and IBA. The mutant, designated *rib1* for *resistant to IBA*, displays phenotypes that are consistent with a primary defect in auxin response or transport.

Materials and methods

rib1 is in the No-0 ecotype of Arabidopsis. All phenotypic characterization of the mutant was done with *rib1* homozygotes derived from the selfed progeny of the original *rib1* isolate. Other mutants used in this paper were obtained from the Arabidopsis Biological Resource Center at Ohio State University.

Plant growth conditions

Seeds were surface sterilized in a solution of 1.5% sodium hypochlorite and 0.02% SDS for five minutes, and rinsed four or five times with sterile water. Seeds were stratified 4 to 7 d in the dark at 4° C before being germinated on Petri dishes containing GM medium containing 0.7% Difco agar. GM medium consists of 1X MS basal salts, 1% sucrose, 0.5g/L MES (2-[N-Morpholino]ethanesulfonic acid), 1 mg/L thiamine, 0.5 mg/L pyridoxin, 0.5 mg/L nicotinic acid, 100 mg/L myo-inositol, with pH adjusted to 5.7 with 1 N KOH (Valvekens et al., 1988). Plated seeds were placed in a growth chamber under the following conditions, unless otherwise stated: 24°C, with a 16h light cycle at a light intensity of 80 to 90 μmol m^{-2} s^{-1}.

For determination of adult phenotypes, crosses, and seed production, seven to ten d-old seedlings were transferred from GM plates to pots containing a 1:1:1 mixture of perlite, vermiculite, and Sunshine mix #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 0.25X Hoagland's solution. Light intensity was approximately 90 μmol m^{-2} s^{-1}.

Isolation of the *rib1* mutant

The *Ac/Ds* transposon tagging system described in Honma et al. (1993) was used to generate a population of 1,419 lines containing independent *Ds* excision events (C.S.W., unpublished results). The selfed progeny of these lines were germinated and

grown on vertically oriented GM plates containing 1.2% Noble agar (Sigma). After 4 d, the plates were rotated 90° on edge. Twenty-four hours later, the plates were scored visually for defects in downward reorientation of the roots. *rib1* was isolated because its root failed to reorient downward (see Fig. 2.1). Selection on chlorsulfuron demonstrated the absence of the *csr1-1* transgene, the selectable marker present in the *Ds* element. Southern analysis with *Ds*-specific probes confirmed that *rib1* mutants do not contain a reintegrated *Ds* element (data not shown).

Genetic characterization and mapping of *rib1*

rib1 homozygotes were crossed to wild-type No-0 plants to determine the genetic basis of the *rib1* mutation. The resulting F1 populations were plated on vertically oriented GM plates containing 0.8% Difco agar and $6x10^{-8}$ M 2,4-D, and scored for resistance by measuring root elongation after 7 d of growth. Wild-type and homozygous mutant seedlings were always included on the same plate with the F1 population being analyzed. Reciprocal crosses were done with *rib1* as the female (three crosses) or the male (six crosses) parent, and all crosses gave the same results; data is presented from the F1 population of a single cross in which *rib1* was the male parent.

F2 mapping populations were generated by crossing *rib1* homozygotes to Columbia wild-type plants. Linkage was examined in both 2,4-D resistant and 2,4-D sensitive individuals. The genotypes of F2 individuals were verified by generating a selfed F3 population for each individual and scoring the 2,4-D resistance of the F3 population. DNA was isolated from pools of 50 F3 seedlings using the protocol of Dellaporta et al. (1983). This DNA was used for PCR reactions to determine linkage of *rib1* to CAPS and SSLP markers covering the Arabidopsis genome. Primers were obtained from Biocorp Inc. (Montreal, Canada) or from Research Genetics (Huntsville, AL, USA). Standard SSLP and CAPS PCR conditions were used (Bell and Ecker, 1994; Konieczny and Ausubel, 1993). Map positions of the markers were taken from the Lister and Dean RI map (http://nasc.nott.ac.uk/new_ri_map.html).

Gravity and slanting response

Seeds were exposed to white light for one to two hours to induce germination and were grown for 4 d in the dark on vertically oriented plates on GM media containing 1.2% Noble agar (Sigma). One plate was scored to determine the root angle at time 0 hour. The other plates were rotated on edge 90° clockwise (when seedlings are viewed through the lid). Individual plates were removed after 2, 4, 6, 8, 10, 12 or 24 hours, digitally scanned, and the angle of each root tip from vertical was measured using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/). No-0 and *rib1* seedlings were plated together to ensure that they were exposed to the same gravity conditions. Similar results were obtained in two separate trials in the dark (data presented), and in three separate trials in the light (data not shown).

Root slanting was measured as the angle from vertical of roots grown for 4 d on vertically oriented GM plates containing 1.2% Noble agar under standard growth chamber conditions. Slanting was scored by viewing the seedlings through the lids of the plates.

Starch determination

Starch accumulation in root tips was examined using the protocol described by Bullen et al. (1990). Iodine and potassium iodide were both obtained from Fisher (Nepean, ON). The starch mutants *pgm-1* and *adg1-1* were used as negative controls. Wild-type and *rib1* seedlings had clearly visible starch granules in their root tips, while none were detectable in *pgm-1* and *adg1-1* root tips.

Hormone and inhibitor response

All hormones and inhibitors were purchased from Sigma, with the exception of NPA, which was purchased from Chem Service (West Chester, PA). MilliQ water, 1 N NaOH, absolute ethanol or DMSO (dimethylsulfoxide) were used to make the appropriate

stocks of these compounds. ABA, IAA, IBA and NAA were dissolved in 1N NaOH and diluted in water to a final stock concentration of 1 to 2 mg/ml. A 1 mg/ml stock of ACC was made in water. All stocks diluted in water were filter sterilized. 2,4-D, HFCA and TIBA stocks were made in ethanol at concentrations of 2, 10 and 0.5 mg/ml, respectively. A 20 mg/ml NPA stock was made in DMSO. Solvent only controls were included for the NPA, HFCA and TIBA assays. Under our conditions, we found that the addition of ethanol up to a concentration of 0.25% did not affect root elongation. Appropriate amounts of the sterile stocks were added to media after autoclaving to obtain the different concentrations required.

Root elongation assays for hormone response were performed as described in Wilson et al. (1990) and for auxin transport inhibitor response as described in Ruegger et al. (1997). In both cases GM medium containing 0.8% Difco agar was used instead of solidified nutrient solution. IAA and IBA response assays were done under yellow long-pass filters (Acrylic yellow-2208, 3.18 mm thick, Commercial Plastics, Montreal) to prevent photodegradation of the auxins (Stasinopoulos and Hangarter, 1990). Wild-type and *rib1* seedlings were transferred to each of the two halves of the same plate, to ensure that they were being exposed to exactly the same conditions. Data are expressed as the percentage of root growth on no hormone or on solvent-only control plates. Similar results were obtained in two to five separate trials. Data from a single representative trial are presented for hormone resistance. A single data point from a representative trial is presented for auxin transport inhibitor resistance.

Root bending assays

Root bending assays were performed as described in Utsuno et al. (1998), except that GM media with 1.5% Noble agar was used instead of solidified nutrient solution. In brief, mutant and wild-type seedlings were germinated on vertically oriented media containing no hormone for 3 d. Mutant and wild-type seedlings were then transferred to vertical plates containing different auxins: 3.3×10^{-8} M IAA, 1×10^{-8} M 2,4-D, 4×10^{-6} M IBA, 6.6×10^{-7} M NAA or to control plates containing no hormone. All plates were then placed under yellow long-pass filters and the root response was scored 2 d later.

Concentrations of IAA, 2,4-D and NAA are those reported by Utsuno et al (1998). We tested three different concentrations of IBA ($1x10^{-6}$ M, $2x10^{-6}$ M and $4x10^{-6}$ M) that were physiologically similar to the concentrations of IAA used; *i.e.*, these concentrations inhibited roots to about the same level in root elongation assays. All three concentrations gave similar results, and we therefore chose to use only the highest concentration for repeat experiments. Mutants and their wild-type parental lines were placed on the same plates to insure that they were exposed to exactly the same concentration of hormone and conditions. The responses of roots to the auxins are either continued growth along the surface of the plate or a sharp 90° turn into the media. This bending phenotype is easily scorable, and the sharp angle of the turn makes it distinct from a normal growth pattern in which a root might penetrate the test medium.

Root length determination

Root length was determined on 7 d-old seedlings grown under our standard conditions. Roots were measured by tracing magnified seedlings using an overhead projector. A transparent ruler placed beside the roots was also traced for use as a scale bar. The tracings were then digitally scanned, and measured using the NIH Image program.

Results

Isolation, genetic characterization, and mapping of *rib1*

Approximately 1,400 *Ds*-mutagenized Nossen (No-0) ecotype lines were screened for mutants with defects in root gravitropism. As seen in Figure 2.1, roots of the *rib1* mutant fail to reorient following a change in the direction of the gravitational vector. We recovered a single line with this phenotype during our screening. Molecular and genetic characterization of *rib1* indicates that this mutation is not tagged by a *Ds* element (see "Materials and Methods" for details).

We have taken advantage of the fact that *rib1* mutants are 2,4-D resistant (see below) for segregation analysis. *rib1* homozygotes were crossed to wild-type No-0 plants. Figure 2.2 shows the 2,4-D resistance of the F1 population (bottom) compared to the two parental groups (top). In this assay, resistance was determined by measuring root elongation on media containing 2,4-D. Homozygous wild-type roots are sensitive to high concentrations of 2,4-D, and were therefore significantly shorter than the *rib1* roots that are 2,4-D resistant. Wild-type seedlings had an average root length of 2.8 ± 0.3 mm (standard error of the mean) compared to an average root length of 4.9 ± 1.1 mm for the homozygous *rib1* seedlings. The distribution of root lengths among the F1 heterozygotes appeared intermediate, with an average root length of 4.3 ± 1.2 mm. We conclude from these data, and from the analysis of the F2 generation (data not shown), that *rib1* is semi-dominant.

We mapped *rib1* to a single locus at the bottom of chromosome I using cleaved amplified polymorphic sequences (CAPS) and simple sequence length polymorphisms (SSLP) markers. *rib1* shows significant linkage to: nga111 at position 1-115.5 (25 recombinants / 84 chromosomes), nF22K20 at position 1-119.5 (21 / 84), and g17311 at position 1-125.4 (18 / 82). *rib1* is not linked to nF5I14 located at position 1-92.1 (data not shown). These data indicate that *rib1* is telomeric to g17311, which maps near the bottom of chromosome I.

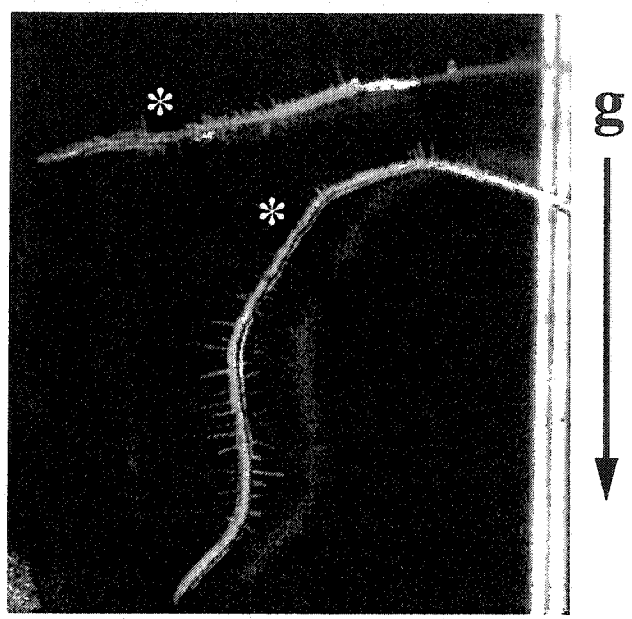

Figure 2.1: Isolation of the *rib1* mutant.
Picture taken during the screen for root gravitropic mutants. Seedlings were grown on vertically oriented plates for four days, then the plates were rotated on edge 90°, and photographed 24h later. The *rib1* root response (top) can be compared to the wild-type root response (bottom). An asterisk indicates the hatch mark showing the position of the root at the time the plate was first rotated. The arrow to the right of the picture indicates the direction of the gravitational vector after rotation. The picture was taken on a Leica stereomicroscope at a magnification of 6.3X.

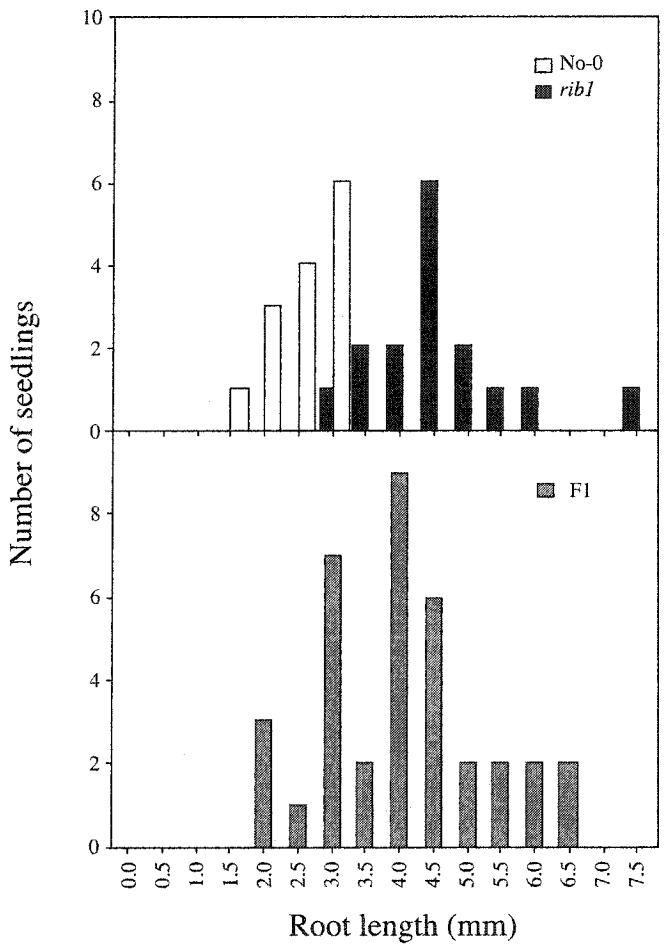

Figure 2.2: Resistance of *rib1* homozygotes and heterozygotes to 2,4-D. Root length distribution of seedlings grown on $6x10^{-8}$M 2,4-D for seven days. A population of F1 seedlings from a No-0 X *rib1* cross (lower panel) is compared to No-0 and *rib1* homozygous populations (upper panel). Statistical analyses (t-test) indicate that *rib1* is not recessive (*P* value = $4.5x10^{-5}$).

Root slanting and gravity response

Although the *rib1* mutant was originally identified based on its altered gravitropic response, *rib1* roots are not agravitropic. When germinated and grown on vertically oriented plates under our standard growth conditions, *rib1* roots are oriented downwards, similar to wild type, and are not randomly oriented as in agravitropic mutants. However, neither wild type nor *rib1* grow directly downwards; instead, they slant to the left of vertical (or to the right when viewed through the agar). Interestingly, *rib1* mutant roots slant more than wild-type roots on vertically oriented plates. The absolute angle values of this slanting response varies from trial to trial, but *rib1* roots usually slant about 20° more to the right than do wild-type roots. For example, in one trial the average angle of *rib1* root tips was 52.4°, while No-0 roots on the same plates had an average root tip angle of 35.9°. The growth of roots aslant from the gravitational vector is characteristic of certain ecotypes of Arabidopsis, including No-0. This response is surface dependent; *i.e.*, it does not occur when roots are embedded in agar or in other conditions where the interaction of roots with the surface of the agar plate are minimized (Rutherford and Masson, 1996; Mullen et al., 1998). The slanting response is thought to be due to interaction between the root and the agar medium itself (chemotropism, hydrotropism, thigmotropism), and also to the effects of gravitropism and the endogenous circumnutation movements of Arabidopsis roots (Okada and Shimura, 1990; Simmons et al., 1995b; Mullen et al., 1998). Reduced gravitropism can explain the root slanting phenotype of *rib1*; roots with a lessened response to gravity would be expected to grow more aslant from the gravitational vector.

Time course experiments were conducted to characterize the defect in gravitational response of *rib1*. These reorientation experiments were done in the dark to ensure that phototropism did not affect the results. Vertically grown seedlings were reoriented by 90° and the curvature of the root tips was measured at different time points over a 24-hour period. The data in Figure 2.3A are presented as the average angle of the root tips from vertical, with error bars representing the SD. During the course of the experiments, wild-type roots (open bars) reorient approximately 60° from their starting average of 100° to an angle approaching 40°. In sharp contrast, the response of the *rib1*

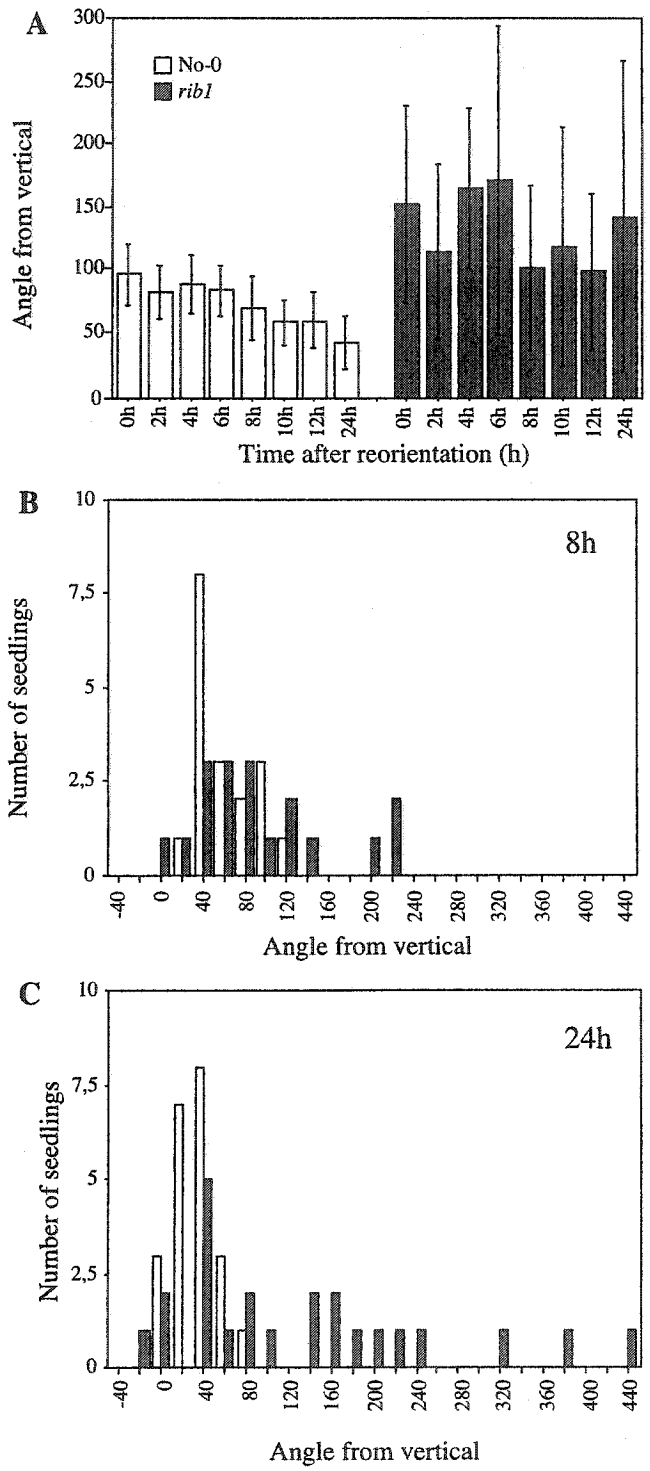

Figure 2.3: Root gravitropic response (Please see figure legend on next page).

Figure 2.3: Root gravitropic response - Figure legend:

Results of time course of turning experiment. Seedlings were grown on vertically oriented plates for four days, then the plates were rotated on edge 90°. The angle of the root tip from vertical was measured after the indicated times. Conventions for root tip angle scoring are as follows: 0° indicates roots that grow directly down towards gravity; 90° is perpendicular to the gravity vector; 180° is directly up away from gravity; >180° represents roots that first turned up and then continued turning to form a loop. Figure 3A represents the average angle from vertical of wild-type (open bars) and rib1 (shaded bars) root tips at different time points. The data for this panel were taken from two combined time course experiments. Error bars represent the standard deviation. Panels B and C present representative time points from one time course experiment. These panels depict a distribution graph of root tip angle at time points 8h (B) and 24h (C).

roots (shaded bars) is much more variable over the 24-h time period, as evidenced by the large SD values. The high average root angles are not indicative of a complete failure of *rib1* roots to reorient, but rather reflect the variability of the *rib1* gravitational response. This point is illustrated in the two representative time points shown in Figure 2.3 B and C, which show the position of individual roots 8 and 24 h after the plates were rotated, respectively. At 8 h, the majority of wild-type roots are reorienting towards 0°, although a few individuals have not yet responded. Similarly, a majority of *rib1* roots are also in the process of reorienting at this time point, but they show a much broader distribution. At 24 h (Fig. 2.3C), wild-type roots are almost fully reoriented, while the *rib1* response is highly variable. Approximately one half of the seedlings show a distribution similar to wild type, while the remaining half are distributed from 140° to 440° from vertical. These results show that *rib1* roots are defective in reorientation following a change in the gravitational vector.

We observed no detectable difference in the level of starch accumulation in *rib1* and wild-type roots (data not shown). This observation, in addition to the fact that *rib1* seedlings show other phenotypes consistent with a primary defect in auxin response or transport (see below), suggest that *rib1* seedlings are most likely affected in gravity response rather than perception.

Morphological characteristics of *rib1*

rib1 seedlings have other root-specific phenotypes, as indicated in Table 2.1. *rib1* seedlings have a shorter (approximately 20%) primary root than wild type and an increase in the total number of lateral roots (approximately 60%). Both of these differences are highly significant: the *P* values of t-tests are 4.5×10^{-6} and 2.6×10^{-9}, respectively. Auxins are known to inhibit root elongation and promote lateral root formation, suggesting that *rib1* seedlings could have a heightened auxin response in the absence of exogenous application of IBA, or a change in the distribution or concentration of this auxin. Soil-grown adult *rib1* plants are visually indistinguishable from wild type. No obvious differences were observed in organ development, flowering time or senescence (data not shown). Quantitatively, we determined apical dominance by counting the number of

Table 2.1: Morphology of wild-type and mutant plants

Characteristic[a]	wild type[b]	*rib1*[b]	Count[c]	t-test *P* value[d]
Root Length (mm)	22.3 ± 0.6	18.7 ± 0.4	62-67	4.5×10^{-6} ***
No. Lateral Roots	21.0 ± 1.1	34.1 ± 1.7	74-76	2.6×10^{-9} ***
Plant Height (cm)	23.3 ± 0.7	22.2 ± 0.7	18-19	0.31
No. Lateral Branches	3.8 ± 0.2	3.5 ± 0.2	18-19	0.22

[a] Root lengths were measured on 7 d-old seedlings, the number of lateral roots was counted on 14 d-old seedlings, and plant height and the number of lateral branches were determined on adult plants.

[b] Values are averages (± S.E.).

[c] Number of seedlings represented by the average.

[d] *** marks a highly significant difference.

primary and secondary inflorescences, and measuring plant height. No differences are seen in these traits between *rib1* and wild-type plants (Table 2.1).

Hormone response

Because many gravitropic response mutants are also auxin resistant, we tested the resistance of *rib1* to different auxins using root elongation assays. These assays are based on the fact that exogenous auxin application inhibits wild-type root elongation as a result of a supra-optimal concentration of this hormone. The results of the assays are presented in Figure 2.4. *rib1* is consistently more resistant than wild type to all concentrations of IBA tested. *t* tests show that these differences are significant in all trials at concentrations that reduce *rib1* root elongation by 15% to 90%. The IBA concentration that inhibits root elongation by 50% (IC_{50}) is about 2.5 fold higher in *rib1* compared to wild type. In contrast to its response to IBA, *rib1* exhibits a wild-type response to IAA. The trial depicted in Figure 2.4 indicates very slight differences between wild-type and *rib1* response for a few concentrations of IAA. Multiple trials have been done and these small differences are not reproducible. We therefore conclude that *rib1* does not display an altered response to IAA. Thus, *rib1* is able to discriminate between the two endogenous auxins of Arabidopsis, IAA and IBA. *rib1* is also more resistant to the synthetic auxin 2,4-D. In the case of 2,4-D the difference in response between *rib1* and wild type is significant at concentrations $5x10^{-8}$ M to 10^{-6} M. *rib1* has a wild-type response to NAA (data not shown), another synthetic auxin. This specificity of resistance makes *rib1* unique amongst the published auxin resistant mutants which are all resistant to both IAA and 2,4-D (Maher and Martindale, 1980; Estelle and Somerville, 1987; Wilson et al., 1990; Hobbie and Estelle, 1995; Leyser et al., 1996; Hobbie et al., 2000). We examined the IBA dose response of the auxin resistant mutants *aux1-7, axr1-3, axr2-1,* and *axr4-2* and found that they are all IBA resistant (data not shown). Therefore, all these mutants are resistant to both endogenous auxins of Arabidopsis.

The response of *rib1* to growth inhibiting concentrations of other classes of plant hormones was also tested utilizing root elongation assays. *rib1* is not more resistant than wild type to the cytokinins, kinetin and benzyladenine (data not shown), to ACC

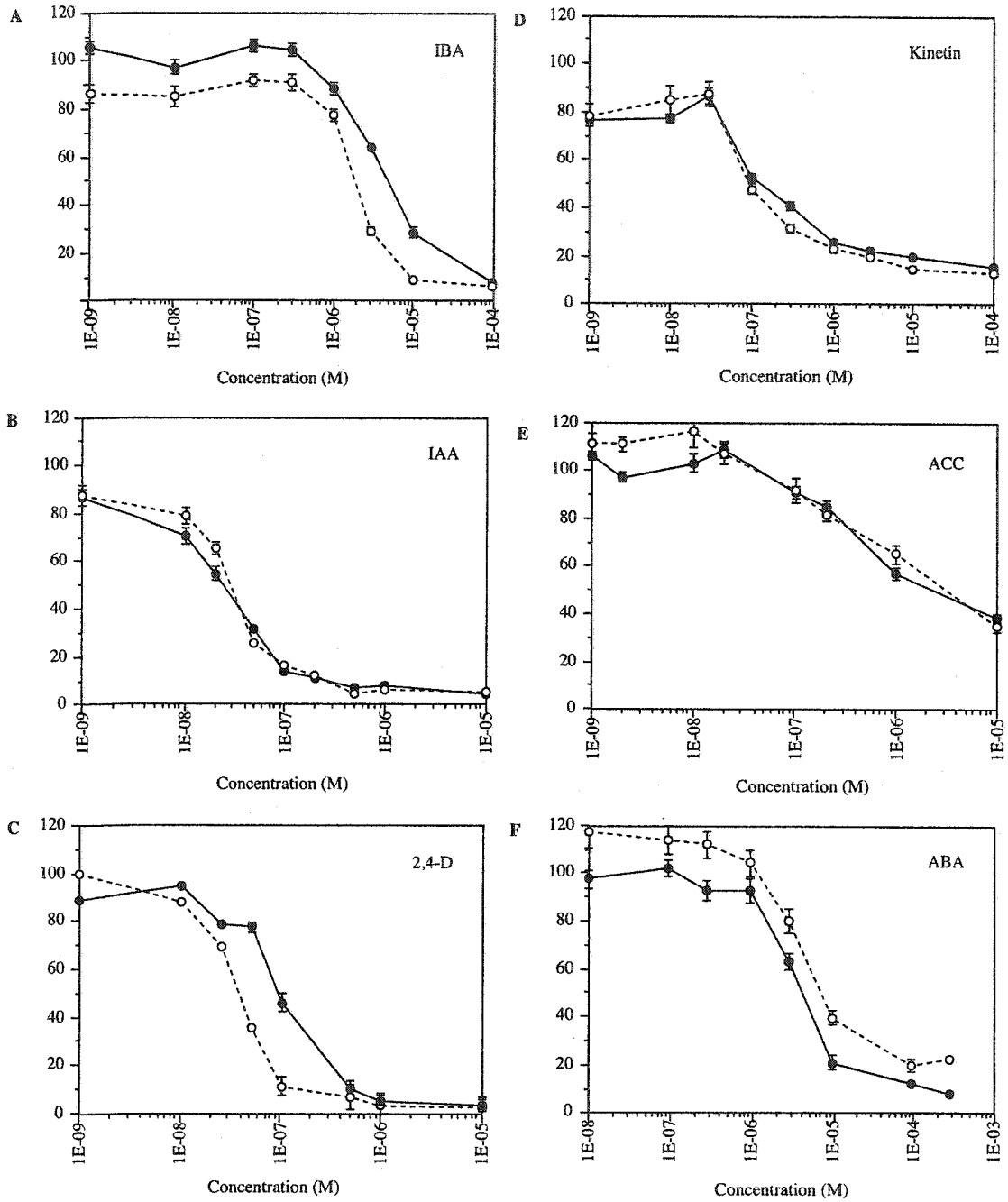

Figure 2.4: Hormone response of *rib1* and wild-type seedlings.
Dose response curves of wild-type (open symbols) and *rib1* (closed symbols) seedling root elongation on IBA, IAA, 2,4-D, Kinetin, ACC, and ABA. Seedlings were grown for five days on GM media and then transferred to media containing the indicated amount of hormones or to control media without hormone. New root growth was measured three days later. Root elongation is expressed as a percentage of root growth on no hormone for each genotype. Each data point represents the average of 13 to 37 seedlings, and the error bars represent the standard error of the mean. Errors smaller than the data point symbols are not indicated.

(1-aminocyclopropane-1-carboxylic acid), the precursor to ethylene, or to abscisic acid (ABA) (Fig. 2.4). The *rib1* mutants show a significant increase in sensitivity to ABA. This difference was significant in all trials at concentrations that inhibit *rib1* root elongation 10% or more. The results presented in Figure 2.4 show that *rib1* is not resistant to any other class of plant hormones.

Resistance to auxin transport inhibitors

The resistance of *rib1* mutants to different auxin transport inhibitors was tested. These compounds all act to block auxin efflux from the cell (Lomax et al., 1995). Figure 2.5 shows the results of root elongation assays in the presence of three different auxin transport inhibitors: naphthylphthalamic acid (NPA, a phytotropin), 2,3,5-triiodobenzoic acid (TIBA) and 9-hydroxyfluorene-9-carboxylic acid (HFCA, a morphactin). *rib1* seedlings are resistant to all three compounds. *t* tests show that this difference is highly significant. These results are consistent with the fact that auxin transport is thought to be important for lateral root initiation, root elongation and root gravitropism (Muday and Haworth, 1994; Lomax et al., 1995).

Root bending assay

The auxin resistance profile of *rib1* mutants shows a striking similarity to findings by Delbarre et al. (1996). These researchers examined the specificity of efflux carriers for different auxins in tobacco suspension cells and suggested that 2,4-D does not utilize the same efflux carrier as do IAA and NAA. Another physiological assay in Arabidopsis roots was recently developed that shows the same auxin specificity (Utsuno et al., 1998). The findings in both studies support the notion that IAA and NAA exit the cell via a specific efflux carrier. Neither group included IBA in their study. We exploited the physiological assay to investigate the role of *rib1* in auxin transport in roots.

The physiological assay as described by Utsuno et al. (1998) uses a clearly visible phenotype (root bending) to study the ability of root cells to efflux auxins. This assay can distinguish wild type from *agr1* by their different responses to exogenous application of

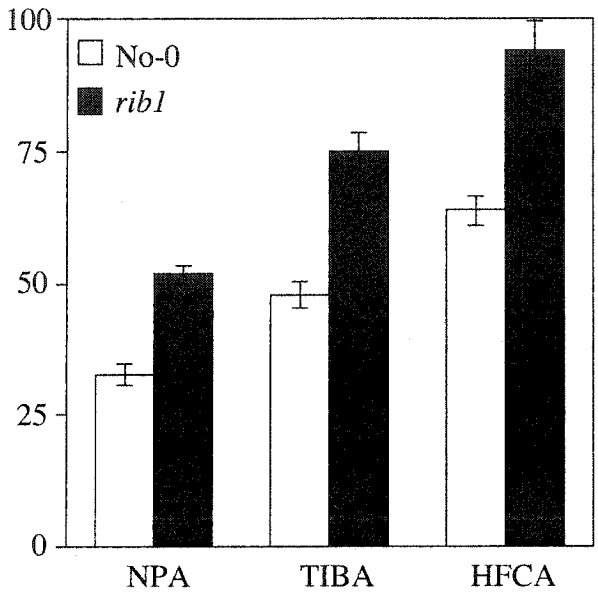

Figure 2.5: Response to auxin transport inhibitors.
Seedlings were grown for three days on GM media and then transferred to media containing the indicated amount of inhibitors or to control media without inhibitor. New root growth was measured five days later. Elongation of roots on 10^{-5} M NPA, TIBA or HFCA is expressed as a percentage of root growth on media containing no inhibitor for both wild type (open bars) and rib1 (shaded bars). Each data point represents the average of 12 to 29 seedlings, and the error bars represent the standard error of the mean.

auxin on one side of the root. AGR1 is expressed in a specific subset of cortical and epidermal cells in the meristematic and elongation zone and is thought to be an efflux carrier important for auxin redistribution in the root. In *agr1* mutants, auxin enters root cells normally, but then accumulates to high levels because of the inability to exit in the absence of a functional efflux carrier. Because the application of auxin in this assay is unilateral, auxin accumulates to inhibitory concentrations on only one side of the root. This results in the root bending phenotype. IAA and NAA elicit the bending response in *agr1*, while 2,4-D does not. This implies that 2,4-D efflux is not dependent on the *AGR1* encoded carrier.

We have performed the bending assay using the *agr1* allele, *eir1-1*, and have obtained the same results reported by Utsuno et al. (1998) for the auxins IAA, NAA and 2,4-D (Table 2.2). The majority of *eir1* seedlings bend their roots into the medium when transferred to plates containing IAA or NAA; 2,4-D or media lacking hormone does not elicit a bending response. Neither wild-type nor *aux1* individuals consistently undergo root bending in response to any of the tested auxins. *rib1* seedlings respond like wild type in this assay on all tested auxins (Table 2.2). A wild-type response on NAA and IAA suggests that *rib1* is not affected in the same efflux pathway as is *eir1*. Likewise, a wild-type response on IBA and 2,4-D suggests that *rib1* is not altering IBA or 2,4-D efflux in the root epidermal and cortical cells in which AGR1 mediates IAA redistribution. However, this does not preclude a role of RIB1 for IBA transport in other root cell types.

Interestingly, IBA fails to elicit root bending in *eir1* seedlings, suggesting that IBA, like 2,4-D, does not use the *EIR1*-encoded efflux carrier. It is noteworthy that the root bending assay results mirror the *rib1* resistance pattern: *rib1* is resistant to IBA and 2,4-D, but not to IAA or NAA, and *eir1* roots bend in response to IAA and NAA, but not to IBA or 2,4-D. These results support the idea that 2,4-D and IBA could behave similarly in terms of transport, in a manner that is distinct from the transport of IAA and NAA. Perhaps another member of the EIR1 family functions as an efflux carrier specific for IBA and 2,4-D transport.

Table 2.2: Root bending assay results

	No auxin	IAA	NAA	2,4-D	IBA
eir1-1[a]	0/21	16/22	21/22	0/21	0/25
WT (Col-O)	0/24	6/20	0/22	0/24	0/28
aux1-7[a]	0/20	1/19	0/20	0/16	0/21
WT (No-O)	0/18	6/21	0/20	0/17	0/24
rib1	0/22	4/23	0/23	0/23	0/26

Data expressed as No. of turned roots/Total No. of roots.

[a] *eir1-1* and *aux1-7* are in the Columbia (Col-0) background.

Discussion

We have isolated and characterized a novel Arabidopsis mutant that exhibits resistance to the natural auxin IBA and to the synthetic auxin 2,4-D, but not to IAA or NAA. This specificity of auxin response is unique amongst published auxin resistant mutants; it demonstrates that *rib1* can discriminate between the two known endogenous auxins of Arabidopsis. Although still considered to be a synthetic auxin by many, IBA was first identified in *Solanum tuberosum* in the 1950s (Blommaert, 1954). In the last decade, investigators have shown that IBA is also present in Arabidopsis and many other species at physiologically relevant concentrations. Despite this, most studies of auxins have focused on IAA as the only natural auxin in plants. The isolation and characterization of the *rib1* mutant provides a genetic tool that can be used to dissect the *in vivo* role of IBA in plants.

Conversion of IBA to IAA has been shown to occur in many different species (Epstein and Ludwig-Müller, 1993). A mutant defective in this process would be resistant to IBA if IBA response is mediated through the conversion of IBA to IAA. While this could be the case for the *rib1* mutant, this model cannot account for the 2,4-D resistance displayed by *rib1*. 2,4-D is an active auxin that does not require modification for activity. In addition, *rib1* seedlings have alterations in root growth that are observed in the absence of IBA treatment. These phenotypes suggest an elevated auxin response and are inconsistent with the reduced production of IAA from IBA predicted by a defect in conversion. Finally, *rib1* does not appear to be defective in β-oxidation, which is the probable mechanism by which IBA is converted to IAA (Normanly et al., 1995). β-oxidation is an enzymatic process that results in shortening of carbon chains by two carbons at a time. Since β-oxidation is also required to mobilize fatty acid reserves during seed germination, mutants defective in β-oxidation grow much more poorly on media lacking sucrose (Hayashi et al., 1998; Richmond and Bleecker, 1999). *rib1* has no requirement for sucrose to promote germination and growth of seedlings in the light or in the dark (data not shown). We conclude from these data that the *rib1* defect does not lie

in conversion of IBA to IAA. Rather, it is likely that the *rib1* mutant phenotypes reflect a change in the ability of seedlings to respond specifically to IBA.

In addition to being resistant to IBA, *rib1* also shows resistance to all auxin transport inhibitors tested by root elongation assays: NPA, TIBA and HFCA. These compounds act principally by blocking carrier-mediated auxin efflux from cells. This inhibition of efflux results in auxin levels that are supra-optimal for root elongation (Muday and Haworth, 1994). For this reason, many auxin response mutants are also resistant to auxin transport inhibitors (Simmons et al., 1995a; Fujita and Syono, 1996; Ruegger et al., 1997). In a similar manner, *rib1* resistance to auxin transport inhibitors is likely to be a secondary consequence of IBA resistance. Early experiments by Leopold and Lam (1961) report that IBA transport in stem segments of sunflower was inhibited by TIBA. Our results support the idea that other IAA transport inhibitors also inhibit IBA transport, resulting in IBA accumulation in cells.

Hormone resistance of *rib1* is limited to specific auxins. The response of *rib1* to cytokinins and ethylene is similar to the wild-type response. However, *rib1* displays a slight increase in sensitivity to ABA compared to wild type. Interaction between different classes of plant hormones is a theme often encountered in plant physiology (Davies, 1995). Ludwig-Müller et al. (1995b) report that either ABA or water stress induces IBA synthetase activity and accumulation of free IBA in maize. The authors propose that the increased IBA levels could mediate the changes in root growth that allow plants to adapt to water stress conditions (Ludwig-Müller et al., 1995b). It is therefore likely that crosstalk between IBA and ABA signaling accounts for the higher ABA sensitivity of *rib1*.

In addition to its altered response to specific hormones and to auxin transport inhibitors, *rib1* also shows other phenotypes in roots; it has a shorter primary root, more lateral roots and a highly variable response to a change in the direction of the gravitational vector. The aerial portion of adult *rib1* plants is indistinguishable from wild type. The phenotypic differences between *rib1* and wild type are most consistent with a primary defect in auxin response or transport in the root.

The *rib1* mutation could affect some component of IBA perception or signal transduction in the roots. *rib1* mutant phenotypes are consistent with a heightened

response to endogenous IBA levels. An increase in lateral root number and a decrease in primary root length can be phenocopied by IBA application to wild-type seedlings (data not shown; Fig. 2.4). This model can also explain the IBA resistance phenotype of *rib1*; *rib1* roots are shorter than wild type because of an increased response to endogenous IBA, and a higher level of applied IBA is therefore required for further inhibition. Similar hypotheses have been proposed to explain auxin resistance caused by semi-dominant mutations in *AXR3/IAA17* and *SHY2/IAA3*, two members of the *Aux/IAA* family of auxin inducible genes (Leyser et al., 1996; Rouse et al., 1998; Tian and Reed, 1999). Both mutants have a shorter primary root in the absence of auxin and are resistant to further root elongation inhibition by auxin. By analogy to the *shy2* and *axr3* mutants, *rib1* could be a gain-of-function mutation in an IBA-inducible gene. We predict that such a gene would be induced by IBA, but not by IAA, treatment. To date, no studies have been done to identify genes specifically induced by IBA. Another possibility is that *rib1* could affect IBA perception or another step in IBA signal transduction. This putative IBA signaling pathway would contribute to the regulation of lateral root formation, root elongation and root gravitropism, phenotypes that are all affected in the *rib1* mutant.

Another explanation for the *rib1* root phenotypes is that this mutation causes an increase in endogenous IBA levels. Although a formal possibility, the phenotypes of *rib1* are not fully consistent with this model. The dominant tryptophan biosynthetic mutant *amt-1/trp5-1* accumulates more tryptophan (Kreps and Town, 1992), and has elevated levels of free IBA (3.5-fold), conjugated IBA (2.5-fold), and conjugated IAA (3.3-fold) (Ludwig-Müller et al., 1993). Like *rib1*, the adult growth pattern of this mutant is reported to be the same as wild type (Kreps and Town, 1992). However, *trp5-1* does not show increased resistance to IBA (data not shown). The root gravitropic defect is also not consistent with an elevated level of auxin; the *sur1* mutant has elevated levels of IAA but displays no defect in root gravitropism (Boerjan et al., 1995). On the other hand, a defect in the control of IBA levels in specific cells or tissues, as opposed to an overall increase in auxin levels as in *trp5* and *sur1*, could result in the *rib1* phenotypes, including altered root gravitropism. Changes in IBA biosynthesis, degradation, conjugation or subcellular compartmentalization could result in localized elevations of IBA levels. Changes in IBA transport could also have the same result.

There is growing evidence that mutations affecting IAA transport have differential responses to various auxins. For example, the *eir1* efflux mutant behaves differently in root bending assays in response to the auxins IAA and NAA compared to 2,4-D. Likewise, the IAA influx mutant, *aux1,* also displays specificity in response to auxins; it is resistant to IAA and 2,4-D, but not to NAA (Yamamoto and Yamamoto, 1998; Marchant et al., 1999). Another example is the *pis1* mutant, a putative regulator of auxin transport, which is specifically hypersensitive to 2,4-D, but not to IAA or NAA (Fujita and Syono, 1997). *rib1*, like *eir1*, *aux1* and *pis1*, has a specific auxin response profile. It is therefore tempting to speculate that *rib1* affects polar auxin transport. In addition, polar auxin transport has been shown to be important for lateral root formation, root gravitropic response and root elongation, which are all processes affected in *rib1* (Lomax et al., 1995).

We cannot at this time define at what step in IBA transport RIB1 might function. A semi-dominant phenotype is consistent with defects in either a regulatory or a structural component of the transport machinery. We are investigating this model further by constructing double mutant lines between *rib1* and the known auxin carrier mutants *aux1* and *eir1*, and by direct assessment of auxin transport in *rib1*.

The recent discovery of multiple homologs of genes encoding auxin efflux and influx carriers suggests that many such carriers exist in plants, each potentially having its own specificity in terms of expression pattern and function (Bennett et al., 1996; Gälweiler et al., 1998). Our root bending assays show that the EIR carrier appears specific for IAA and NAA transport and is not required for IBA and 2,4-D transport. This is also consistent with the results of Delbarre et al. (1996), which show that 2,4-D does not use the IAA/NAA-specific efflux carrier expressed in tobacco cell cultures. We suggest that efflux carriers also exist for IBA transport. The differential expression of this hypothetical IBA carrier, in conjunction with that of the IAA carriers, would provide exquisite control over the levels of both endogenous auxins throughout the plant.

This hypothesis might appear to contradict another conclusion of Delbarre et al. (1996), who suggested that 2,4-D efflux would be mostly carrier independent. However, carrier independent efflux is inconsistent with the fact that 2,4-D undergoes polar transport, since it is the basal localization of the efflux carrier that determines the polarity

of transport. To explain the results of Delbarre et al., we suggest that the hypothetical IBA/2,4-D efflux carrier is not present in tobacco cell cultures such as those used in their study. However, the carrier would be expressed in specific, differentiated tissues such as the root.

We have isolated other IBA resistant mutants that are currently being characterized (J.P. and C.S.W., unpublished data). Similarly, other researchers have also recently isolated IBA resistant mutants (Zolman et al., 2000). Amongst these, two mutants were recovered that, like *rib1,* are resistant to IBA and 2,4-D, but not to IAA. These mutants map to separate loci from *rib1*. Therefore, the specificity of auxin resistance demonstrated by these mutants may represent a new class of auxin resistant mutants. The small number of these mutants recovered to date, together with the fact that no allelic mutations have been recovered, indicate that the genome has not yet been saturated for this class of mutants. Characterization of new IBA-specific mutants will allow us to further understand the activity of this endogenous auxin in plant development. The phenotypes displayed by *rib1* imply an *in vivo* role of IBA in roots and seedlings. Phenotypic analysis of new alleles of *rib1*, as well as cloning and sequencing of the gene, should provide insights into the function of the RIB1 protein in the plant.

Acknowledgements

The technical assistance of Sharon Chen in the initial stages of this work is gratefully acknowledged. We wish to thank Marc Trudel for help with statistical analysis, Dr. G. K. Muday, A.V. Babwah and A. J. Windsor for helpful comments on the manuscript, and Dr. B. Bartel for communicating results prior to publication. Mutant seeds stocks were obtained from the Arabidopsis Biological Resource Center at Ohio State University.

Linking paragraph between Chapters 2 and 3:

The phenotypes displayed by the *rib1* mutant discussed in the previous chapter, the shorter primary root, increased number of lateral roots and the defects in gravitropic response, could all be explained by a defect in IBA transport in this mutant. In addition, many IAA transport mutants have differential responses to various auxins, just as *rib1* does. To further explore this possibility, it was necessary to first analyze IBA transport in wild-type Arabidopsis, as this transport was not sufficiently well characterized in Arabidopsis, seedlings or adult plants. This is the subject of the following chapter.

Chapter 3: IBA transport in wild-type plants

Transport of the Two Natural Auxins, IBA and IAA, in Arabidopsis

Rashotte, AM, Poupart, J, Waddell, CS, Muday, GK (2003) Plant Physiology, Vol. 133, pp. 761-772

Running Title: Transport of Two Natural Auxins

Transport of the two natural auxins, IBA and IAA, in Arabidopsis

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Abstract

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.

Introduction

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-Müller et al., 1993). Although there have been great advances in understanding the molecular mechanisms behind IAA action and transport (Leyser, 2002; Muday and DeLong, 2001; Friml and Palme, 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-Müller, 2000; Bartel et al., 2001). IBA has been identified in a number of plant species from maize (*Zea mays)* and peas (*Pisum sativa)* to Arabidopsis, and concentrations of free IBA approach the levels of free IAA in a number of plants (Ludwig-Müller, 2000). IBA, like IAA, is also found in conjugated forms, yet at significantly lower levels than IAA (Ludwig-Müller 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; Zolman et al., 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, as it is much more potent than IAA or synthetic auxins (Ludwig-Müller, 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 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 following 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 (see for example Wiesman et al., 1988; van der Krieken et al., 1992; Epstein and Lavee, 1984; 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-Müller et al., 1995c). Using a nonstandard assay, transport of both IAA and IBA in inflorescence axis was found to occur mostly in the acropetal direction, but with some basipetal transport of both auxins (Ludwig-Müller et al., 1995c). 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-Müller et al., 1995c). 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-Müller et al., 1995c) 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 naphthylphthalamic acid (NPA), have 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 towards the base (basipetal transport).

To detect auxin movements in seedlings, tritiated auxin was applied from a 1mm 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 transport (RAT), 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 Tables 3.1 and Figure 3.1. 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 hours after application in either a basipetal or an acropetal manner (Table 3.1; data not shown). To determine if low levels of IBA transport could be detected in the inflorescence axis, an additional pulse-chase assay was

Table 3.1: 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 $P > 0.05$.

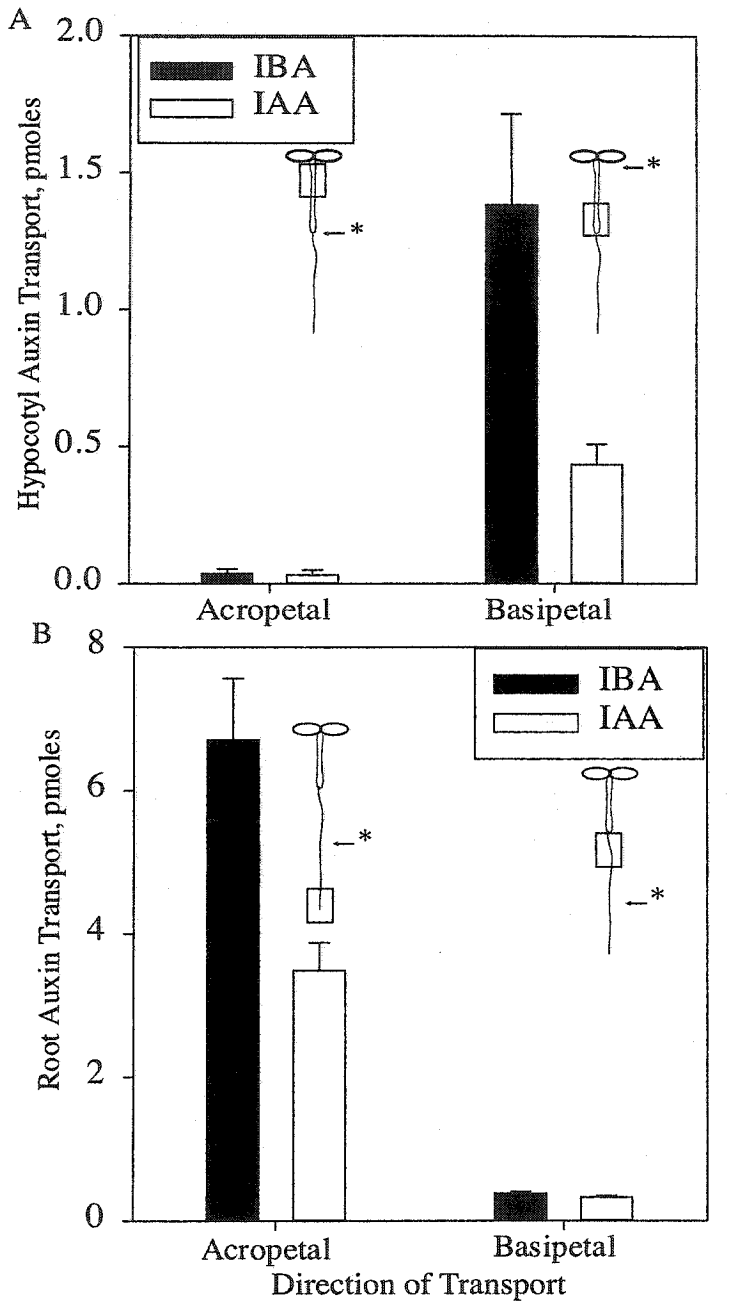

Figure 3.1: Polarity of IBA and IAA transport. A. Direction of IAA and IBA movement in hypocotyls was examined by applying ^{3}H-IBA or ^{3}H-IAA at the base or the tip of the hypocotyl and measuring radioactivity in distant 5-mm segments, as shown on inset. (±SE; n=30). B, Acropetal and basipetal movement 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 on 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.

used, since 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. 3.2). 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. 3.1A). 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. 3.1B). Because this site of auxin application is behind the zone of basipetal auxin transport, both 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 towards the base was quantified (Fig. 3.3A). 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

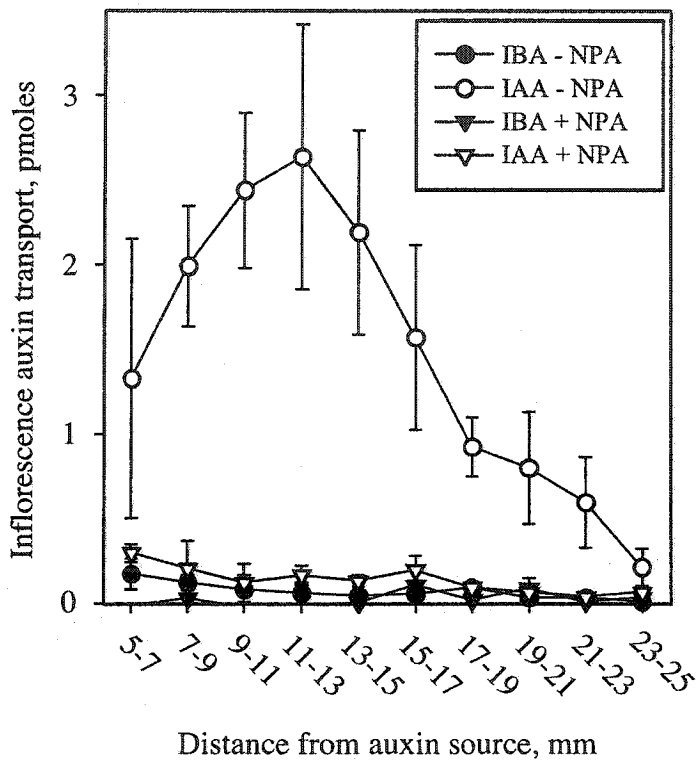

Figure 3.2: Inflorescence axis transport of IBA and IAA. IBA and IAA basipetal transport was examined in 25-mm inflorescence axis segments. The apical end of each segment was placed in ^{3}H-IBA or ^{3}H-IAA for 10 min followed by a 90 min chase of the respective unlabeled auxin, either with or without NPA. The radioactivity in 2-mm segments was determined. (±SE; n=3)

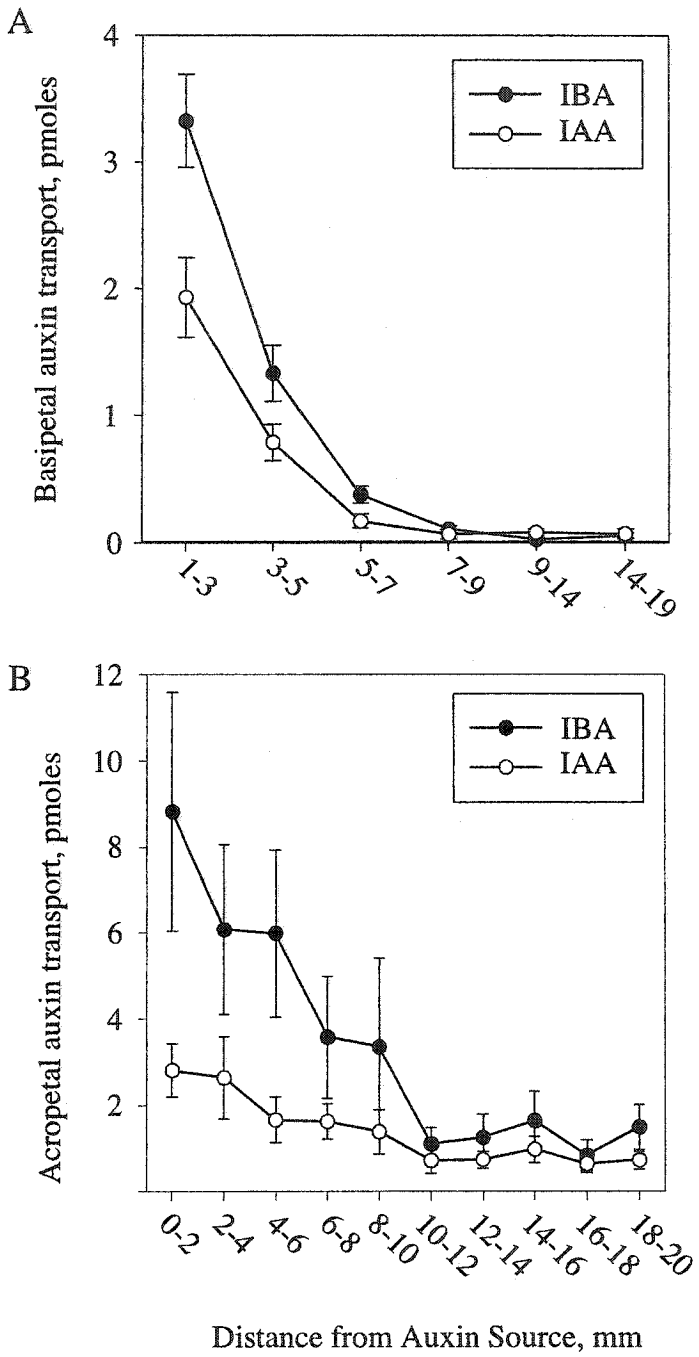

Figure 3.3: Distance and rate of IAA and IBA transport. A, Distance of IBA and IAA basipetal transport from the root tip was examined by applying ^{3}H-IBA or ^{3}H-IAA at the root tip and measuring radioactivity in either 2- or 5-mm segments spanning the indicated distance from the root tip. B, Rate 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)

measure the rate of acropetal transport than basipetal 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. 3.3B). 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 3.1 and 3.2). 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 $\pm$ 0.2 pmoles) as compared to untreated controls (4.5 $\pm$ 0.5 pmoles), but did not significantly affect IBA movement (12.2 $\pm$ 1.4 pmoles) as compared to controls (11.4 $\pm$ 1.4 pmoles). 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.

Table 3.2: 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.

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 previously shown 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* and in the wild-type Columbia background are shown in Table 3.3. Basipetal IAA transport is significantly reduced in *aux1-7* and *eir1-1,* and acropetal IAA transport is reduced in *aux1-7.* We have previously found 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 3.3, 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 unlabelled IBA or IAA were added to an agar cylinder containing a constant level of radioactive auxin in a RBT assay (Fig. 3.4). 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.

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

Table 3.3: 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

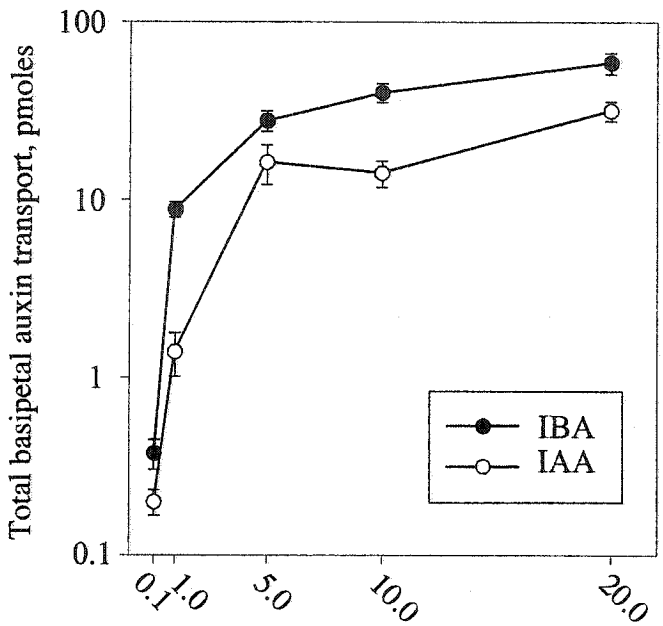

Figure 3.4: Saturation of auxin transport. The ability of IBA and IAA transport to saturate RBT was measured as a function of the movement of a constant amount of ^{3}H-IBA or ^{3}H-IAA with increasing unlabeled amounts of the respective auxin in distant 5-mm segments. (±SE; n=10).

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 3.5 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.

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 3.2. 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 3.2) and the IAA transport is no longer sensitive to NPA.

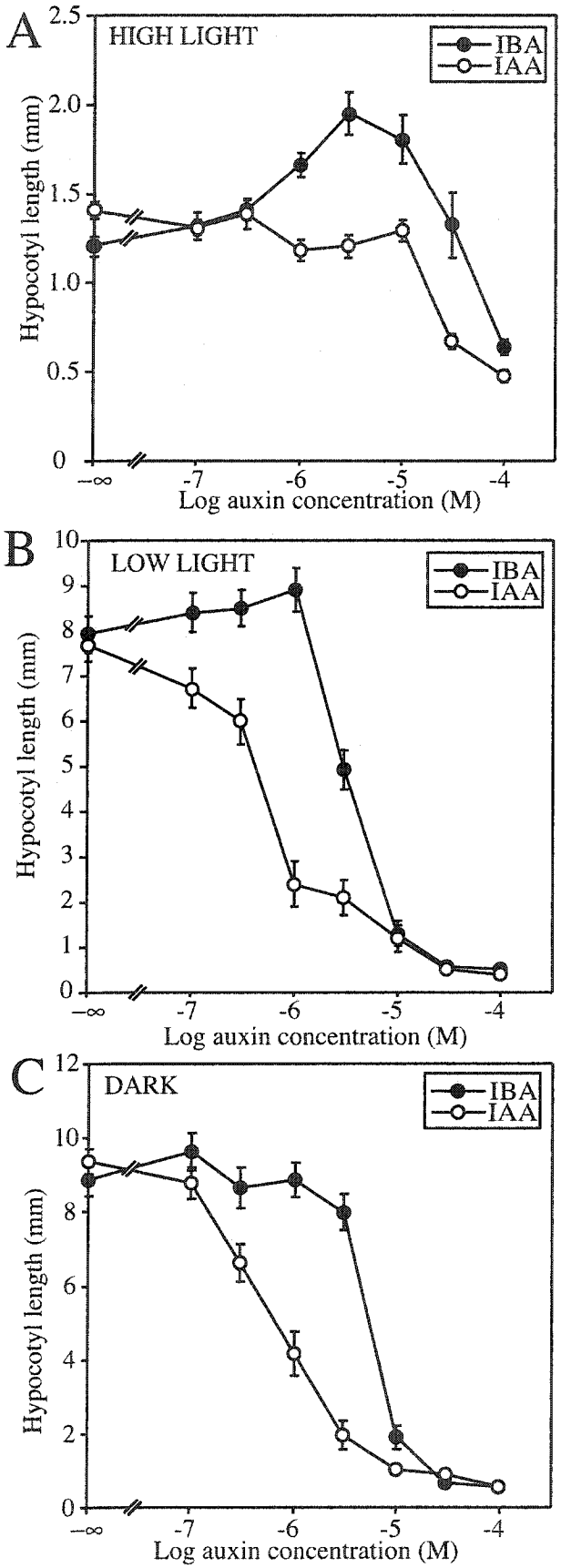

Figure 3.5: Effect of auxins on hypocotyl elongation. Dose response curves for hypocotyl elongation in response to 5 d on IAA or IBA under high white light (90 μmol $m^{-2}s^{-1}$; A) , low light (5 μmol $m^{-2}s^{-1}$; B) or dark (C) conditions are reported. (±SE; n=6 to 26)

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-Müller et al., 1995c). 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-Müller et al., 1995c). 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-Müller et al. (1995c) was suggested to be movement in the transpiration stream and it is unclear whether the basipetal auxin movements reported in that work were truly polar transport.

In order to look for IBA transport in our system, we performed several assays for time periods spanning from 5 to 36 hours, 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 previously reported.

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, while 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. As 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 recently implicated 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-Müller (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 3.3B, 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 prior to 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, as 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 minutes to 24 hours, 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; Kepinski and Leyser, 2002; Muday, 2001). 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-D 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, since 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-Müller et al., 1995c). These results can be reconciled by the hypothesis that IAA, but not IBA, is transported into the cell through the AUX1 carrier, while 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-Müller et al., 1995c). 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 to 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 may therefore 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 previously examined. 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 Arabidopsis 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 sensitive, 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 to 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-Müller 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 (Zolman et al., 2001b; Richmond and Bleecker, 1999). 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 auxin 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), 3-[5(n)-^{3}H]-Indole acetic acid (27 and 25 Ci mmol^{-1}) from Amersham (Arlington Heights, IL). 3-[^{3}H(G)]-Indole butyric acid (25 Ci mmol^{-1}) was prepared in a custom synthesis under conditions designed to label the indole ring by American Radiolabeled Chemicals (St. Louis, MO). All other chemicals were obtained from Sigma (St. Louis, MO).

Seed germination and plant growth

Wild-type *Arabidopsis thaliana* 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 minutes and surface sterilized with 95% ethanol for 5 minutes and 20% bleach with 0.01% Triton X-100 for 5 minutes. After 5 washes in sterile distilled water, seeds were germinated and grown on 9-cm Petri plates containing sterile control medium containing 0.8% agar (Sigma type M, plant tissue culture), 1X Murashige and Skoog salts, pH 6.0; 1.5% (w/v) sucrose; 1 μg mL^{-1} thiamine; 1 μg mL^{-1} pyridoxine HCl; 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 mixture of perlite, vermiculite, and Sunshine mix #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 X Hoagland's 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 days.

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 hours. 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 non-radioactive auxin of similar concentration for 90 min. Higher concentrations of radioactive auxins were used in the pulse chase experiments, since 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 root basipetal transport (RBT) and on top of the seedlings, just on the root side below the root shoot junction for root acropetal transport (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. 3.2B), both 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. Root acropetal transport 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. 3.3B), 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 non-radioactive 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 3.4, 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 mL 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 mL of concentrated hydrochloric acid were added to the bleach, and the desiccating jar was quickly closed. Seeds were left to sterilize for three to six hours, 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 four to seven days in the dark at 4°C before being germinated. For growth analyses only, IAA and IBA were dissolved in 1 ml of 1N NaOH and diluted with 49 mls of dH2O 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 medium (GM) used in these studies is buffered (see below), addition of the stocks did not result in any pH change in the medium.

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 1X MS basal salts, 1% (w/v) sucrose, 0.5 g/l MES (2-[N-Morpholino]-ethanesulfonic acid), 1 mg thiamine, 0.5 mg L^{-1} pyridoxin, 0.5 mg L^{-1} nicotinic acid, 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 (approx. 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 NIH 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 sterile distilled water and blotted dry. The seedlings were transferred to a new microfuge tube containing 200μl 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). Unlabelled 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 ; 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 non-radioactive 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 5ml 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 to mutant or inhibitor treated seedlings or to compare IAA and IBA treatments in physiological assays. The *P* values are reported.

Acknowledgements

We appreciate the assistance of Mary Beth Lovin and Shari Brady exploring some unanswered questions and members of the Muday lab for support and critically reading the manuscript. We also thank Alison DeLong (Brown University) for her critical comments on the manuscript. This work was supported by grants from the National Aeronautics and Space Administration Specialized Center for Research and Training (North Carolina State University) to A.M.R. and G.K.M, NASA Grant NAG2-1507 to GKM, J. W. McConnell McGill University Majors Fellowship to J.P. and Natural Sciences and Engineering Research Council of Canada to C.S.W.

Linking paragraph between Chapters 3 and 4:

In Chapter 3, we characterize IBA transport in Arabidopsis seedlings and adult plants, and compare it to the transport of IAA. We show that there are many similarities between IBA and IAA transport, but that differences also exist, and that these differences suggest different mechanisms might mediate or regulate the transport of the two auxins. That paper is the groundwork permitting the analysis, in the next chapter, of the transport of IBA in the *rib1* mutant, which was shown in Chapter 2 to be specifically defective in response to IBA, while retaining wild-type sensitivity to IAA. This mutant displays phenotypes consistent with the hypothesis that it could be defective in IBA transport.

Chapter 4: Auxin transport in *rib1*

The *rib1* mutant of Arabidopsis has alterations in IBA transport, hypocotyl elongation and root architecture

(Poupart, J, Rashotte, AM, Muday, GK, Waddell, CS, (2004)
Manuscript in preparation

Running Title: Modified IBA transport in *rib1*

The *rib1* mutant of Arabidopsis has alterations in IBA transport, hypocotyl elongation and root architecture

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This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada to C.S.W., the J. W. McConnell McGill University Majors Fellowship to J.P. and grants from the National Aeronautics and Space Administration Specialized Center for Research and Training (North Carolina State University) to A.M.R. and G.K.M, and NASA Grant NAG2-1507 to G.K.M.

Abstract

Polar transport of the auxin IBA has recently been shown to occur in Arabidopsis seedlings, yet the physiological importance of this process has yet to be fully resolved. In this study, we look at the transport of IAA and IBA in the *rib1* mutant, which is defective in IBA response but has a wild-type response to IAA. One hypothesis formulated to explain *rib1* phenotypes proposed a defect in IBA transport, and the results presented here confirm that IBA transport is altered in *rib1*. There is a decrease in IBA hypocotyl basipetal and root basipetal transport in *rib1* and an increase in IBA root acropetal transport. IAA transport levels are not different in *rib1* compared to wild-type, but sensitivity of root acropetal IAA transport to the inhibitor NPA is abolished in the mutant. The changes in IBA transport can be correlated with *rib1* phenotypes. The mutant has a shorter primary root, increased lateral root formation and modifications in hypocotyl elongation are seen in different light conditions. We found sucrose affected the hypocotyl elongation response to light in *rib1*. Finally, we investigated the effects of exogenous application of auxins or NPA on hypocotyl elongation and lateral root formation. Taken together, our results suggest modifications in IBA distribution in seedlings affect hypocotyl and root elongation, and lateral root formation, supporting a model in which endogenous IBA could influence seedling morphology.

Introduction

The auxin indole-3-butyric acid (IBA) occurs naturally at levels that are physiologically relevant in many plant species, including Arabidopsis (reviewed in Ludwig-Müller, 2000). Its auxin activity is well recognized, as it has long been used as the auxin of choice for root formation on cuttings (Zimmerman and Wilcoxon, 1935; Hartmann et al., 1997). Like the auxin indole-3-acetic acid (IAA), IBA affects lateral root induction, root and shoot elongation and hypocotyl elongation (Yang and Davies, 1999; Poupart and Waddell, 2000; Zolman et al., 2000, Rashotte et al., 2003). Polar auxin transport is a specialized delivery system that moves IAA from its point of synthesis in young apical tissues to the rest of the plant in a highly regulated manner (Bennett et al., 1998). IBA, like IAA, is transported in a polar fashion, in roots and hypocotyls of Arabidopsis, but, unlike IAA, IBA is not transported in inflorescence stems (Rashotte et al., 2003). The transport of IBA is regulated differently than that of IAA; IBA transport is not sensitive to inhibition by auxin efflux inhibitors, and the *agr1/eir1* and *aux1* mutants, that are defective in IAA transporters, have wild-type levels of IBA transport (Rashotte et al., 2003). These results suggest an IBA transport pathway distinct from the IAA transport pathway exists.

Proper regulation of auxin transport is important for plant growth and development. Disruption of IAA polar transport using auxin efflux inhibitors such as NPA (naphthylphthalamic acid) and TIBA (triiodobenzoic acid) results in a variety of phenotypes including defects in embryo and vascular tissue patterning, inhibition of lateral root, leaf primordia and floral organ formation, reduced hypocotyl elongation in the light, and altered root gravitropism. (Okada et al., 1991; Muday and Haworth, 1994; Hadfi et al., 1998; Jensen et al., 1998; Mattsson et al., 1999; Sieburth, 1999; Reinhardt et al., 2000). Many mutations that disrupt auxin transport and affect the above-mentioned aspects of plant development have been isolated, and proteins involved in auxin transport have been identified through analysis of such mutants. Three different families of putative carrier proteins have been identified, that are products of the *AUX, PIN* or *MDR* gene families (Noh et al., 2001, and review in Luschnig, 2002). Regulatory proteins have also been identified, such as those encoded by *RCN1* or *PID1*, and flavonoids have been suggested to act as endogenous regulators of auxin efflux (Deruère et al., 1999;

Christensen et al., 2000; Brown et al., 2001). The current model is that expression of different carriers and regulatory proteins in specific cells allows precise regulation of auxin transport in a tissue and cell-specific manner (reviewed in Muday and DeLong, 2001). It has also been suggested that different transporters could have different substrate specificities or affinities (Luschnig, 2002). In addition to these endogenous factors, exogenous factors also affect auxin transport, such as light and gravi-stimulation.

The suggestion that light influences auxin transport was made as early as 1880 by the pioneering work of Charles Darwin and his son Francis, who described a diffusible influence (which was later identified as auxin) moving from the tip of an illuminated coleoptile to the elongation zone (Darwin and Darwin, 1880). Links between auxin transport and photomorphogenesis in seedlings have also been supported by recent publications. Redistribution of auxin following tropic stimulation has been shown by the activation of an auxin inducible reporter gene on one side of stems, coinciding with differential growth (Li et al., 1991). Dim red light was shown to cause an increase in polar auxin transport in cucumber hypocotyls (Shinkle et al., 1998). Steindler and co-workers have shown that shade-induced hypocotyl elongation was dependent on auxin transport, as it can be inhibited by NPA, and the auxin response mutant *axr1-12* is defective in this response (Steindler et al., 1999). These results suggest light can affect auxin transport. The recent cloning of the *BIG* gene also supports a link between auxin transport and light response. Surprisingly, it was determined that two Arabidopsis mutants, *doc1* (Dark overexpressor of CAB), which was isolated because it expresses abnormally high levels of light-induced genes in the dark, and *tir3* (transport inhibitor response), which is defective in auxin transport and in NPA binding, are mutated in the same gene, named *BIG*. It was shown that increasing auxin in *tir3* or *doc1* mutant backgrounds using the auxin biosynthetic mutant, *yucca*, can suppress expression of most light-induced genes in the dark. This suggests that a defect in auxin distribution in the dark is the cause of aberrant gene expression in *doc1* and *tir3*. Analysis of this and other phenotypes of BIG mutants suggests this protein is involved in regulation of auxin transport. In this case auxin transport is required in dark-grown seedlings to repress light-induced gene expression.

Many mutants that are defective in auxin response also have defects in photomorphogenesis. For example, dominant mutations in the *AXR2/IAA7*, *AXR3/IAA17* and *SHY2/IAA3* auxin response genes all cause short hypocotyls, expanded cotyledons and leaf production in the dark, phenotypes which are characteristic of photomorphogenesis and not seen in etiolated wild-type seedlings (Leyser et al., 1996; Tian and Reed, 1999; Nagpal et al., 2000). HY5 is a bZIP protein that is suggested to act as a positive regulator of light signaling. *hy5-1* mutants not only have defects in hypocotyl elongation in the light, but also in root gravitropism and lateral root formation. As these root phenotypes are reminiscent of those of auxin-related mutants, it has been suggested that HY5 could be involved in a pathway mediated by auxin and that it may regulate expression of auxin-inducible genes (Oyama et al., 1997). Interestingly, mutations in *HY5* (a positive regulator of light response) and *COP1* (a negative regulator of light response) result in converse and antagonistic defects in lateral root formation: *hy5-1* mutants have more lateral roots, while *cop1-6* mutants lack lateral roots (Ang et al., 1998). These auxin-related phenotypes in light response mutants further suggest a functional link between the light and auxin signaling pathways.

In this paper, we studied IAA and IBA transport in different tissues of the *resistant to IBA (rib1)* mutant. *rib1* was isolated in a screen for mutants with defects in root gravitropism, and shown previously to have an altered response to a change in the direction of the gravity vector. Further characterization of the mutant revealed it was resistant to root elongation inhibition by the auxin IBA, and the synthetic auxin 2,4-D, but had a wild-type response to IAA and the synthetic auxin NAA. *rib1* did not show resistance to other classes of plant hormones tested, but did show resistance to the IAA efflux inhibitors NPA, TIBA and HFCA. Phenotypically, *rib1* seedlings could be distinguished from wild-type by the fact they had a shorter primary root and more lateral roots. Adult *rib1* plants however were indistinguishable from the wild-type in terms of shoot length and secondary inflorescence formation. Based on its phenotypes and the resistance of *rib1* to IAA efflux inhibitors, we had hypothesized that this mutant could be defective in IBA transport or response (Poupart and Waddell, 2000).

This study shows that *rib1* is defective in IBA transport, but has wild-type levels of IAA transport. This is, to the best of our knowledge, the first demonstration of such a

phenotype. These defects in IBA transport in seedlings can be correlated to defects in root elongation, lateral root formation and hypocotyl elongation. Our results suggest an implication of IBA in hypocotyl response to light signals, and that this response could be modulated by sucrose. Finally, we also present the characterization of the responses of *rib1* and wild-type seedlings to exogenous IAA and IBA, and to auxin transport inhibitors NPA and TIBA. Our results suggest RIB1 could be a regulator of IBA transport important for defining root architecture and in hypocotyl elongation response to light.

Results:

Transport of auxins in *rib1*

In seedlings, both IAA and IBA are transported in three different flows: from the tip of the hypocotyl down towards the root (hypocotyl basipetal transport), from the base of the root to the tip (root acropetal transport) and from the root tip back up towards the root base over a short distance (root basipetal transport) (Muday and DeLong, 2001, Rashotte et al, 2003). We measured IAA and IBA transport in *rib1* and wild-type seedlings to directly assess the impact of the *rib1* mutation on auxin transport (Table 4.1). Significant changes in IBA transport are seen in all three pathways. In *rib1* seedlings, hypocotyl basipetal transport of IBA is reduced to approximately 60% of the value of wild-type, while IBA root basipetal transport is reduced approximately 25% relative to wild-type. Converse to the decreases observed for basipetal transport, root acropetal IBA transport is increased relative to wild-type levels approximately 1.8-fold.

In contrast to the alterations in IBA transport, no significant alteration in IAA transport was observed in any of the seedling assays (Table 4.1). In addition, IAA basipetal transport in inflorescence stems occurred normally in *rib1* adult plants, and IBA did not undergo detectable amounts of transport (Table 4.1). These results are consistent with the wild-type appearance of adult *rib1* plants.

Effect of NPA on IAA transport in WT and *rib1*:

Though overall levels of IAA transport are unaffected by the *rib1* mutation under the conditions of our assays (Table 4.1), analysis of the effects of NPA on IAA transport reveals a difference in regulation of IAA transport in *rib1* relative to wild-type. Table 4.2 shows that all flows of IAA transport are significantly reduced by NPA in wild-type seedlings and adult plants. Likewise, IAA basipetal transport in the inflorescence stem, hypocotyl and root are inhibited by NPA in *rib1*. In contrast, NPA does not inhibit root acropetal IAA transport in *rib1*.

Table 4.1: Transport of IAA and IBA in wild-type and *rib1* tissues

	pmoles transported[a]		
Transport Assay	WT	*rib1*	*P* value[b]
Hypocotyl Basipetal			
IAA[c]	3.56 ± 0.35	3.21 ± 0.22	0.40
IBA[c]	3.08 ± 0.32	1.79 ± 0.20	1.51×10^{-3}
Root Basipetal			
IAA[d]	2.02 ± 0.12	1.79 ± 0.09	0.14
IBA[e]	9.90 ± 0.64	7.40 ± 0.45	1.66×10^{-3}
Root Acropetal			
IAA[f]	9.27 ± 0.64	10.39 ± 0.64	0.22
IBA[f]	17.11 ± 0.89	30.44 ± 1.39	1.55×10^{-13}
Inflorescence Basipetal			
IAA[g]	1.12 ± 0.09	1.31 ± 0.12	0.22
IBA[g]	0.04 ± 0.005	0.04 ± 0.006	0.67

[a]Average and standard error of the mean are presented.

[b]Two-tailed Student's t-test assuming equal variance

[c] n values are from 30 plants from 3 experiments

[d] n values are from 134-136 plants from 14 experiments

[e] n values are from 59 plants from 6 experiments

[f] n values are from 79-82 plants from 7-8 experiments

[g] n values are from 14-15 segments from 3 experiments

Table 4.2: Effect of NPA on IAA transport in wild-type and *rib1* tissues

	pmoles transported[a]			
	HBT[c]	RAT[d]	RBT[e]	IBT[f]
WT - NPA	3.43±0.39	11.37±1.31	2.30±0.18	1.12±0.09
WT + NPA	1.38±0.26	7.95±0.42	1.44±0.12	0.03±0.01
P value[b]	2.0×10^{-10}	7.1×10^{-3}	4.8×10^{-5}	6.0×10^{-13}
rib1 - NPA	3.34±0.35	13.38±1.13	2.01±0.15	1.31±0.12
rib1 + NPA	1.31±0.18	15.04±1.18	0.88±0.09	0.05±0.02
P value[b]	1.2×10^{-13}	0.16	1.4×10^{-9}	7.4×10^{-11}

[a]Average and standard error of the mean are presented.

[b]One-tailed Student's t-test assuming equal variance for the presence and absence of NPA

[c] HBT: Hypocotyl basipetal transport, n values are from 29-30 plants from 3 experiments

[d] RAT: Root acropetal transport, n values are from 28-35 plants from 3-4 experiments

[e] RBT: Root basipetal transport, n values are from 65-66 plants from 7 experiments

[f] IBT: Inflorescence basipetal transport, n values are from 14-15 segments from 3 experiments

Hypocotyl elongation in *rib1*:

As there is a significant decrease in hypocotyl basipetal transport of IBA, we therefore wanted to determine whether hypocotyl elongation was affected in the *rib1* mutant. Under the conditions used for the transport assays, low white light (5 μmol m^{-2} s^{-1}) and 1.5% sucrose, *rib1* hypocotyls are about 20% longer than wild-type hypocotyls (Table 4.3). In the dark on the same medium, *rib1* hypocotyls are approximately 35% longer than wild-type. However, there is no difference in hypocotyl length under high white light conditions (95 μmol m^{-2} s^{-1}). This last result was surprising as we had noted previously that *rib1* had a long hypocotyl under our standard growth conditions (Poupart and Waddell, 1998); this prompted us to further examine the effects of light on hypocotyl length under different conditions.

We looked at hypocotyl elongation under no sucrose conditions. Table 4.4 shows the length of hypocotyls in mutants and wild-types under different light conditions. In the absence of sucrose, *rib1* hypocotyls are significantly longer than wild-type in white (approximately 30% longer) and red light (approximately 45% longer), but not in the dark or blue light conditions. There is also a small, yet significant, reduction (5%) in hypocotyl elongation under far-red light conditions in *rib1* relative to wild-type. Our results suggest a modification in IBA transport is associated with defects in hypocotyl elongation reminiscent of those of phytochrome mutants. As salt conditions were the same for experiments presented in Tables 4.3 and 4.4, the only significant difference between these media was the presence of sucrose. This difference results in an important change in hypocotyl elongation in *rib1* relative to wild-type: *rib1* hypocotyls are longer in the dark or low light in the presence of 1.5% sucrose, and longer in high light in the absence of sucrose. Surprisingly, in the presence of both high light (95 μmoles m^{-2} s^{-1}) and sucrose, *rib1* hypocotyl lengths are similar to wild-type (Table 4.3). These results uncover a difference in the combined effects of light and sucrose on hypocotyl elongation in *rib1*.

Table 4.3: Hypocotyl elongation in wild-type and *rib1* on media containing 1.5% sucrose

Light condition	WT	*rib1*	*P* value [a]
Low White (5μmoles m^{-2} s^{-1})	8.92 ± 0.16[a]	10.35 ± 0.17	3.11 X 10^{-9}
High White (95μmoles m^{-2} s^{-1})	1.45 ± 0.03	1.36 ± 0.03	0.051
Dark	10.46 ± 0.19	14.12 ± 0.20	2.53 X 10^{-32}

Average length in mm and SE of 138-209 individuals from 7-9 trials are presented.

[a] Two-tailed Student's t-test *P* values are reported.

Table 4.4: Hypocotyl elongation in wild-type and *rib1* on media lacking sucrose

Light condition	WT	*rib1*	*P* value[a]
High White	1.62 ± 0.08[a]	2.14 ± 0.09	3.89×10^{-5}
Dark	16.75 ± 0.30	16.25 ± 0.37	0.31
Red	7.01 ± 0.22	10.26 ± 0.24	1.75×10^{-15}
Far red	2.85 ± 0.04	2.71 ± 0.05	0.04
Blue	3.18 ± 0.01	3.38 ± 0.08	0.12

Average length in mm of 32 to 143 individuals ± standard error of the mean are presented.

[a]Two-tailed Student's t-test assuming equal variance

Effect of exogenous auxins on hypocotyl elongation:

The effects of exogenous IAA and IBA on hypocotyl elongation were investigated in three different light conditions: high white light, no light, and the low light conditions used for the hypocotyl basipetal transport assays. Medium with 1.5% sucrose was used, as this is the medium used in auxin transport assays (Figure 4.1). In the dark both IAA (Fig. 4.1A) and IBA (Fig. 4.1B) inhibit elongation of *rib1* and wild-type hypocotyls. Small but significant differences in response of *rib1* to IAA under dark conditions were detected at concentrations of 10 μM and 30 μM (Fig. 4.1A). More notably, the dose response curve of *rib1* to IBA is clearly shifted towards higher concentrations, indicating a reduced sensitivity of *rib1* to IBA under these conditions (Fig 4.1B). Highly significant differences in response at 3 μM and 10 μM were found.

Under low light conditions, the *rib1* dose response curves to both IAA (Fig 4.1C) and IBA (Fig 4.1D) are shifted towards higher concentrations relative to the wild-type curve, indicating that hypocotyl elongation in *rib1* is resistant to inhibition by auxins in these conditions also. For IAA response, significant differences are seen at concentration points from 0.1μM to 1μM. In the case of IBA, differences seen at concentrations at and above 1μM are significant. Some differences are however more pronounced for IBA, as can be seen by the larger distance between the dose response curves of wild-type and *rib1*.

IBA significantly stimulates hypocotyl elongation under high light at concentrations from 1 μM to 10 μM in the wild-type (Fig. 4.1F), as previously reported (Rashotte et al 2003). At higher concentration (100 μM), IBA inhibits hypocotyl elongation in wild-type. In high light, higher amounts of auxins are required to inhibit hypocotyl elongation, compared to low light and dark conditions (compare the dose response curves in 4.1 E and F to those of Fig.4.1 A to D). Inhibition of wild-type hypocotyl elongation requires approximately 30 μM of IAA or 100 μM IBA in high light versus 0.3 μM to 1μM IAA or 3 to 10 μM IBA in dark and low light conditions. In high light, though the wild-type and *rib1* dose response curves to IAA mostly overlap, there is a small but statistically significant difference in response at a concentration of 30 μM, where *rib1* shows low level resistance to IAA (Fig. 4.1 E). This difference was

Figure 4.1 - Figure legend on next page

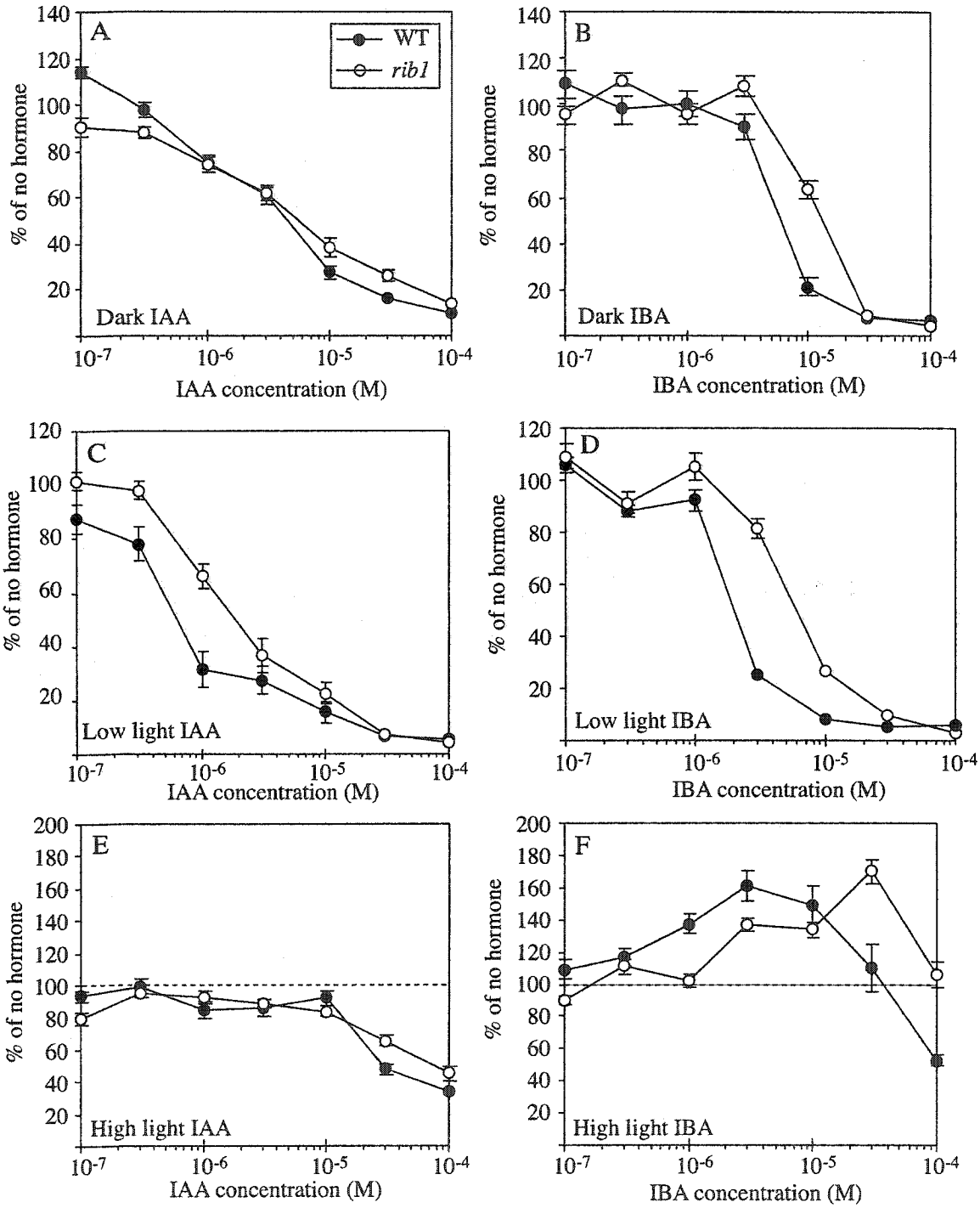

Figure 4.1 – Figure legend:

Dose response of hypocotyl elongation to auxins in different light conditions in the presence of 1.5% sucrose. Wild-type (closed symbols) and *rib1* (open symbols) seedlings were plated on media containing the indicated amounts of IAA (panels A, C, E) or IBA (B, D, F). Plates were placed in dark (A, B), low light (C, D) or high light (E, F) conditions, and hypocotyl length was measured after 5 days of growth. Average and SE is presented as the percent of elongation in the absence of auxin. Errors smaller than the symbols are not presented. The average of 6-26 seedlings from one representative trial is shown. Similar results were obtained in two or three other trials.

significant in two of three trials. In these conditions, the dose response curve of *rib1* to IBA (Fig. 4.1 F) is again shifted towards higher concentrations relative to the wild-type curve. This can be seen by the fact that a higher concentration of IBA is required not only to stimulate elongation (1 μM results in longer wild-type hypocotyls, while 3 μM is required to stimulate *rib1*), but also to inhibit elongation (wild-type is significantly inhibited at 100 μM IBA, while the *rib1* curve has not yet fallen below the 100% line).

Effect of exogenous auxins on lateral root formation:

Lateral root initiation in response to exogenous auxin was investigated in wild-type and *rib1*. We first tested the effects of IAA and IBA on wild-type seedlings of the Nossen ecotype; Figure 4.2 shows a dose response curve of lateral root formation in response to IAA and IBA in this ecotype. Lower concentrations of IBA than IAA are required to stimulate lateral root initiation, and the IBA curve is therefore shifted towards lower concentrations. Approximately 10 fold higher concentrations of IAA than IBA are required to reach 50% of the maximum effect. The differences in root inducing capacity between IAA and IBA were shown to be significant by *t*-tests at data points between 0.3 μM and 10 μM, suggesting that IBA is a more potent inducer of lateral roots than IAA under these conditions.

Figure 4.3 shows dose response curves of lateral root initiation in wild-type and *rib1* in response to IAA (Fig. 4.3 A) and IBA (Fig.4.3 B). IAA stimulates lateral root formation in *rib1* and wild-type in a similar fashion, as can be seen by the fact the wild-type and *rib1* curves approximately follow each other. This contrasts with the results seen for IBA, in which the wild-type and *rib1* curves are well separated. A maximal level of induction of lateral root formation in wild-type of approximately 13-fold is reached in wild-type at an IBA concentration of 10 μM. At the same concentration, IBA induces only a 5.3-fold increase in lateral root formation in *rib1*, indicating that though *rib1* is not fully resistant to lateral root induction by IBA, it is still less sensitive to this effect of IBA. The induction response seems to have reached a plateau in wild-type, as higher concentrations of IBA do not significantly increase lateral root formation. In contrast, in

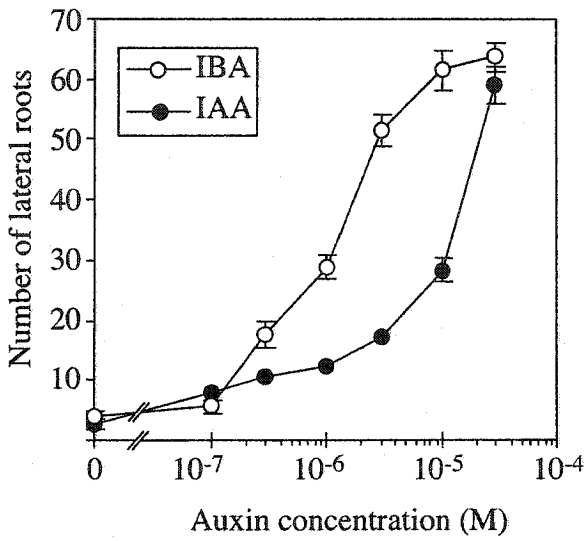

Figure 4.2: Comparison of IAA and IBA effects on lateral root numbers in wild-type seedlings. Dose response curves of the effects of IAA (closed symbols) or IBA (open symbols) on lateral root number. Four-day-old seedlings were transfered to media containing the indicated amounts of auxin. The average number of lateral roots formed after 6 days of growth in the presence of auxin is reported with SE. Each value is the average of 17 or 18 individual seedlings. Errors smaller than the symbol for each point are not indicated.

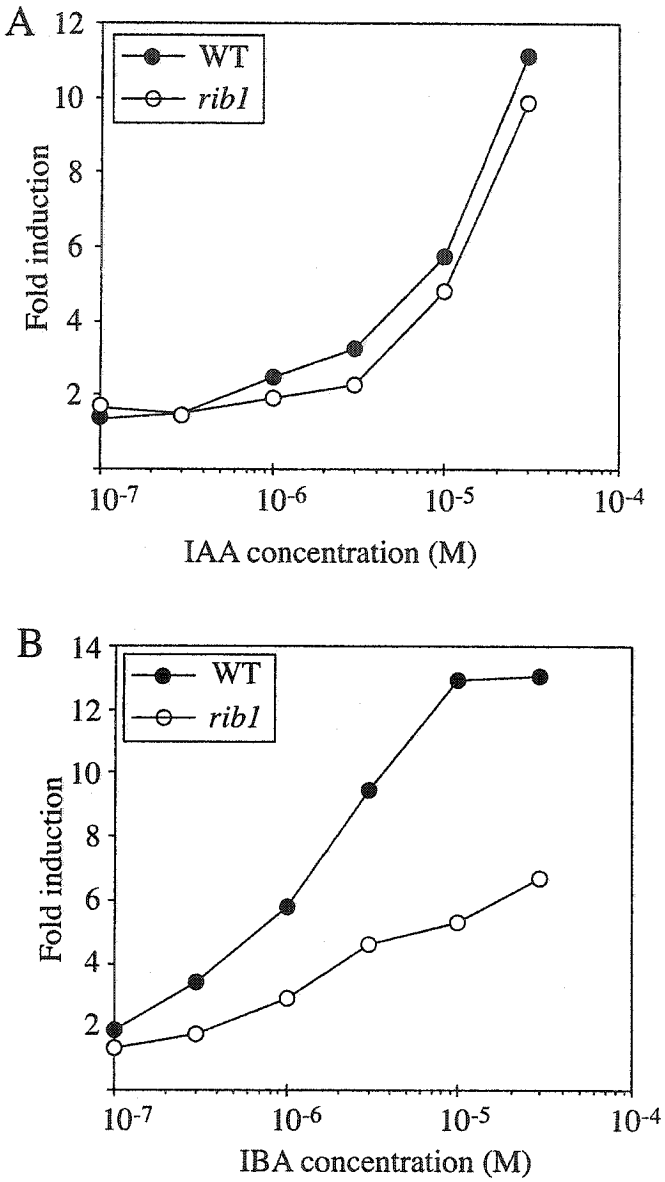

Figure 4.3: Effect of auxins on lateral root formation in wild-type and *rib1* seedlings. Dose response of lateral root initiation in wild-type (closed symbols) and *rib1* (open symbols) in response to IAA (A) or IBA (B). Four-day-old seedlings were transfered to media containing the indicated amounts of auxin. Lateral roots formed after 6 days of growth in the presence of auxin were counted, and the data is presented as fold-increase in root number relative to growth on unsupplemented media. The average of 17 or 18 seedlings from one representative trial is shown. Similar results were obtained in two other trials.

rib1, increasing the levels of IBA results in a higher number of lateral roots, suggesting the response is not yet maximal in the mutant at the concentration used.

Effect of auxin transport inhibitors on *rib1* seedlings:

The *rib1* mutant has been shown to be resistant not only to IBA and 2,4-D, but also to the auxin efflux inhibitors NPA, TIBA and HFCA by root elongation assays (Poupart and Waddell, 2000). Figure 4.4 shows a picture of wild-type (left side) and *rib1* (right side) seedlings grown for 14 days on vertically oriented plates in the presence of 0.1μM NPA. NPA at this concentration abolishes normal gravity response in both wild-type and *rib1* seedlings, but differences in response of the two genotypes can also clearly be seen. As previously reported, root elongation in wild-type is more sensitive to inhibition by NPA, and this results in short thick roots in the majority of wild-type seedlings (Fig. 4.4 and data not shown), while *rib1* produces much longer roots. The swelling of root tips, which is a characteristic response to auxin transport inhibitors (Ruegger et al., 1997), was only seen in wild-type, and not in *rib1,* seedlings. Additionally, hypocotyls of wild-type seedlings were much shorter than those of *rib1* seedlings under these conditions.

Figure 4.5 shows the effects of the auxin efflux inhibitors NPA and TIBA on lateral root initiation in wild-type and *rib1* seedlings. The concentration of NPA used (0.1μM) inhibits lateral root formation to approximately 20% of the numbers in non-treated wild-type seedlings. In contrast, NPA reduces lateral root formation in *rib1* much less, to about 77 % of non-treated levels. *rib1* is also resistant to the effects of TIBA: at 0.1μM, TIBA reduces the number of lateral roots formed in wild-type to approximately 45 % and in mutant to 71% of non-treated controls. Interestingly, in wild-type NPA is significantly more effective in inhibiting lateral root formation than TIBA (20% vs. 45%; t test *P* value of 6 X 10^{-3}), while the difference between the effect of 0.1μM NPA or TIBA is not significant in *rib1* (77% vs. 71%; t test *P* value of 0.66). Taken together, these results show that *rib1* is resistant to auxin efflux inhibitors for both primary root elongation and lateral root formation responses.

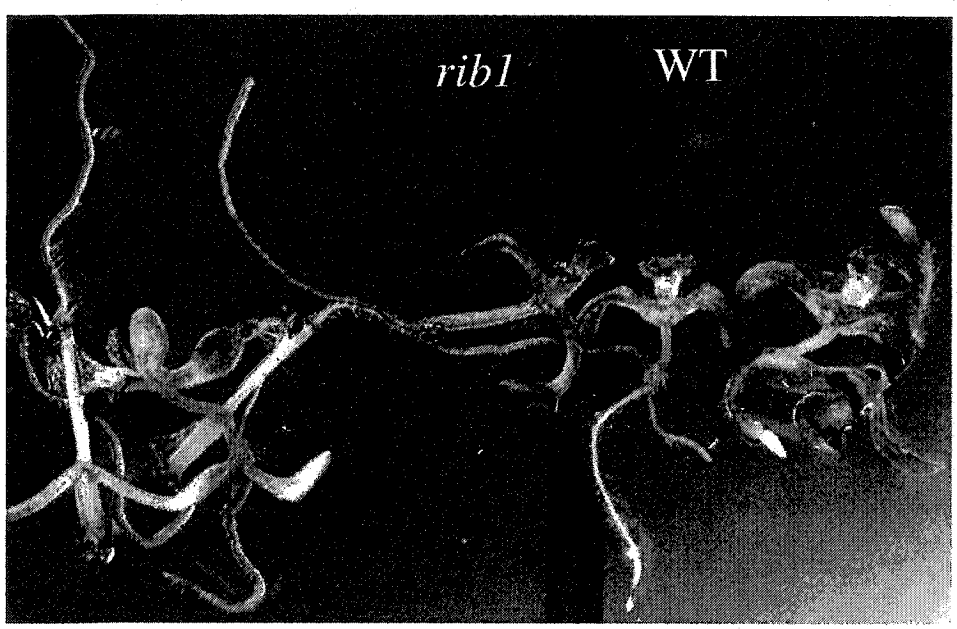

Figure 4.4. Picture of the effects of NPA on wild-type (right) and *rib1* (left) seedlings. Seeds were plated directly on vertically oriented plates with media containing 0.1μM NPA and a picture was taken after 14 days of growth in continuous high light.

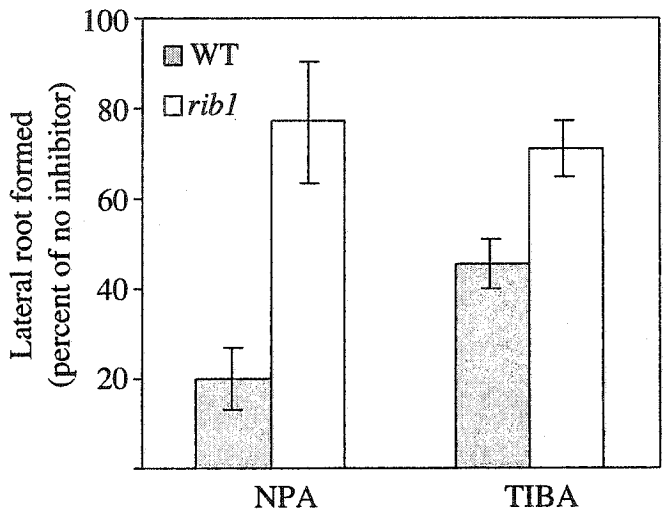

Figure 4.5: Effect of auxin transport inhibitors on lateral root formation in wild-type and *rib1* seedlings. Wild-type (filled bars) and *rib1* (open bars) seedlings were grown for 4 days on unsupplemented media and then transfered to media containing 0.1μM NPA or TIBA. The number of lateral roots formed was counted 6 days later, and data is expressed as a percentage of the number of lateral roots formed in the absence of inhibitor. Average and SE of 36 individuals from two assays (NPA) or 54 individuals from 3 assays (TIBA) are presented.

Discussion

Disruptions in IAA transport caused by inhibitor treatment or mutation has helped dissect the role of the different flows of this auxin in plants. Though IBA has recently been shown to be transported in a polar fashion in Arabidopsis, the role or importance of this transport remains to be defined precisely (Rashotte et al., 2003). As the so-called auxin efflux inhibitors do not affect IBA transport, other means must be used to study the transport of this endogenous auxin. The current study shows that all flows of IBA transport are affected in *rib1* seedlings, while IAA transport levels are unchanged relative to wild-type. We therefore suggest that analysis of the phenotypes and auxin response profiles of this mutant can help dissect out the role of IBA transport in Arabidopsis, and other plants.

Changes in IBA transport in *rib1* can be linked to specific modifications in root and hypocotyl morphology, and these phenotypes are reminiscent of those of seedlings in which IAA transport has been disrupted. Table 4.5 summarizes these results. For example, blocking root basipetal transport of IAA by localized application of auxin transport inhibitors, or by mutations in transport proteins such as AGR1/EIR1/PIN2/WAV6 or AUX1, results in defects in gravity induced reorientation and root elongation (Maher and Martindale, 1980; Bell and Maher, 1990; Rashotte et al., 2000). Because of this, it has been suggested that root basipetal transport of IAA is required for proper elongation responses of wild-type roots (Rashotte et al., 2000). *rib1* mutants have a slowed response to gravity and a shorter primary root, indicating a defect in elongation growth, but do not have a defect in IAA transport. They do however show a reduction in IBA root basipetal transport suggesting a role for this auxin in regulating root elongation growth. Similarly, root acropetal transport of IAA has been shown to be important for lateral root formation, as inhibition of this flow of transport results in a reduction of lateral root formation (Reed et al., 1998b). *rib1* mutant seedlings have approximately 60% more lateral roots than wild-type at 14 days (Poupart and Waddell, 2000), and this is consistent with the higher root acropetal transport of IBA reported here. The root phenotypes of *rib1* (increased number of lateral roots, shorter primary root and slowed gravity response) are therefore suggested to be the result of IBA transport

Table 4.5: Summary of results[a]

Transport flow	Hypocotyl basipetal	Root acropetal	Root basipetal
Physiological role	hypocotyl elongation	lateral root initiation	elongation/gravitropic response
Effect of applied IBA	inhibits elongation	induces initiation	high conc. inhibit elongation
Modification in IBA transport in *rib1*	40% decrease	1.8X increase	25% decrease
rib1 phenotypes	long hypocotyl	more lateral roots	short primary root, decreased gravity response

[a]See discussion for details.

disruptions, indicating that IBA, in addition to IAA, has an important role in defining root architecture.

Hypocotyl basipetal transport of IBA is reduced in the *rib1* mutant, while IAA transport is unaffected in this tissue. We also found hypocotyl elongation to be affected by this mutation in a range of conditions. In the absence of sucrose, *rib1* hypocotyls are longer in white and red light, not significantly different under dark or blue light, and shorter under far-red light. The long hypocotyl phenotype of *rib1* is consistent with a defect in a PhyB-mediated response, since in *phyB* mutants, as in *rib1*, longer hypocotyls in white and red light are seen (Reed et al., 1993). The fact that *rib1* presents shorter hypocotyls in far-red light (5% shorter, t-test *P* value 0.04) suggests, on the other hand, increased signaling from another phytochrome, PhyA. We found the reduction in *rib1* hypocotyl length in far-red light to be even greater in the presence of 1% sucrose (18% shorter, t-test *P* value 1.22 X10-19, data not shown). It is interesting to note that overexpression studies show PhyB is a sucrose-dependent dominant negative suppressor of PhyA mediated inhibition of hypocotyl elongation; PhyB overexpressing lines have longer hypocotyls in far-red light in the presence of sucrose, but not in its absence (Short, 1999). Our results also suggest interaction between PhyB and PhyA signaling, and sucrose also has an important effect on hypocotyl elongation of *rib1*. Further studies will be required to define exactly how sucrose and phytochrome signaling pathways interact, and the role of auxin transport in mediating signals from these sources.

The *rib1* phenotypes suggest a defect in translating the light signal perceived by phytochromes into an appropriate response. In the current model for molecular regulation of photomorphogenesis, the light signal perceived by photoreceptors is transduced by early regulatory factors specific to each photoreceptor; a signal integration step then follows resulting in suppression of negative regulators of photomorphogenesis such as COP1 and DET1, and activation of positive regulators of photomorphogenesis such as HY5 and many others, in a complex regulatory web (recently reviewed in Quail, 2002). Exactly where and how RIB could be integrated in this complex web is unclear at present, but it would affect IBA transport in response to light. Light has been shown previously to affect auxin transport in cucumbers (Shinkle et al., 1998), but also to specifically affect IBA transport in Arabidopsis (Rashotte et al., 2003).

In the presence of sucrose, *rib1* hypocotyls are longer than wild-type in dark and low light conditions, but not different in high light conditions. Our data therefore suggest IBA transport has a role in hypocotyl elongation both in the light (without sucrose) and in the dark (with sucrose). This conclusion may seem to contradict the results of Jensen et al. (1998), which suggested auxin transport was important for hypocotyl elongation in the light only, as NPA could inhibit elongation in light, but not in the dark. These results can be reconciled if we consider the specificities of IBA transport; analysis of IBA transport in Arabidopsis has shown it is transported in the same directions as IAA, but that it is not sensitive to inhibition by NPA. Therefore, lack of inhibition by NPA cannot be invoked to rule out the implication of IBA transport. Additionally, IAA transport itself is not completely abolished by NPA in hypocotyls, unlike what is observed in inflorescence stem, where NPA causes complete inhibition of IAA transport (Rashotte et al., 2003). This suggests some IAA transport also occurs through an NPA insensitive pathway. This NPA insensitive IAA transport could use the same pathway as IBA transport. Interestingly, Jensen et al. (1998) noted that NPA had a lesser effect on hypocotyl elongation in red light than in white, far-red or blue light conditions. Based on this result and results presented here, it is tantalizing to suggest that NPA insensitive transport is more important for the PhyB-mediated hypocotyl elongation inhibition response.

Exogenous auxin has different effects on hypocotyl elongation depending on light intensity. Stimulation of hypocotyl elongation in high light is seen in wild-type with IBA concentrations of 1 to 10 μM, a reaction not seen under dark or low light conditions. Stimulation is also seen in *rib1* hypocotyls with IBA concentrations approximately 3-fold higher. The fact that IBA can stimulate hypocotyl elongation suggests this auxin has a role in elongation of this organ in Arabidopsis. Under the high sucrose and nutrient conditions used in this study, no stimulation of hypocotyl elongation by IAA was seen. This result is in agreement with the results of Smalle et al. (1997), who showed IAA could stimulate hypocotyl elongation on nutrient-deficient growth media, but not in rich media (defined as 0.5X strength MS salts, 1% sucrose; media used for IAA and IBA response assays are even richer: 1X MS salts, 1.5% sucrose). Under low white light or dark conditions, application of either IAA or IBA results in inhibition of hypocotyl elongation.

Overall, the effects of applying low concentrations of IBA in the presence of sucrose are the opposite of those caused by the *rib1* mutation for hypocotyl elongation. *rib1* seedlings have longer hypocotyls in conditions under which application of low concentrations of IBA were shown to inhibit elongation (dark and low white light), but show hypocotyl lengths similar to wild-type under high white light conditions, where application of the same concentration of IBA had no significant effect on elongation. In high white light conditions, approximately 10-fold higher concentrations of exogenous IBA are required to inhibit hypocotyl elongation than in other light conditions. The hypocotyl elongation phenotypes seen under dark, low light and white light conditions are therefore all consistent with a reduction in IBA levels in *rib1* hypocotyls: such a reduction would relieve inhibition under dark and low light conditions, making hypocotyls longer, but have a non significant effect in high white light conditions, consistent with the higher concentration of IBA required to affect hypocotyl elongation in these conditions. The reduced transport of IBA measured in *rib1* hypocotyls could result in reduced IBA levels. Direct measurements of IBA in seedlings would however be necessary to confirm this hypothesis.

Additionally, our results demonstrate clearly that media conditions (in our case presence or absence of sucrose) have a profound effect on light response in *rib1*. Cross talk between sucrose and phytochrome signaling pathways have previously been demonstrated. In addition to the above-mentioned amplification by sucrose of PhyB effects on PhyA signaling, other recently isolated light response mutants also show sucrose dependent phenotypes. For example, the *shygrl1* mutant, which is defective in phytochrome mediated induction of a subset of light inducible genes, presents a long hypocotyl phenotype and increased anthocyanin accumulation only when grown in the light on media containing sucrose. Interestingly, *shygrl1* has wild-type hypocotyl length in the absence of sucrose, so it presents the converse of the hypocotyl defects of *rib1* in high light (Santiago-Ong et al., 2001).

Cross-talk of IBA with other hormone response pathways is also possible. *rib1* is more sensitive to ABA mediated inhibition of root elongation than WT (Poupart and Waddell, 2000). Many recent studies have shown ABA signaling to be required for

normal sucrose sensitivity (reviewed in Sheen et al., 1999; Gibson, 2000). Exactly how IBA, sucrose and ABA signaling pathways could intersect is unclear at present.

A further link between light, sucrose and auxin effects can be made in the case of IBA. IBA is known to act at least partially through conversion to IAA. IBA to IAA conversion occurs through the process of peroxisomal ß-oxidation, and therefore, many other IBA resistant mutants have defects in peroxisomal proteins (Zolman et al., 2000). ß-oxidation is also used by plants to release energy from stored fatty acids during dark growth, while in the light, the main energy source is photosynthesis. Accordingly, light results in down-regulation of ß-oxidation genes (Ma et al., 2001). How this affects IBA to IAA conversion has, to our knowledge, not been investigated, but is an intriguing possibility.

In this context, it is also interesting to note that the *cop1*, *det1* and *ted3* mutants, that are thought to be central regulators of light response, are all resistant to IBA (Hu et al., 2002). Though this is suggested to result from defects in peroxisomes in these mutants it will be interesting to see how this affects the balance of auxin in seedlings, and might help better understand the role/effects of IBA in light regulated development. As it is still unclear whether IBA acts directly as an auxin or if it must be converted to IAA for activity, it would also be interesting to determine if some of the defects in gravitropism or lateral root formation in the *hy5* and *cop1* mutants could be due to IBA accumulation (in *cop1* mutants) or changes in IBA response (in *hy5* mutants).

IBA was a more potent inducer of lateral roots than IAA in wild-type under the low light conditions used for transport and physiological assays. These results contrast with the results of Zolman et al. (2000) who found that IAA induced more lateral roots than IBA at similar concentrations. Many factors vary between our studies and these have been shown to affect lateral root formation in Arabidopsis: age of seedlings, both sucrose and salt composition of media used (Malamy and Ryan, 2001), length of treatment, ecotype and light conditions likely account for this difference. *rib1* is resistant to lateral root induction by IBA, but not by IAA, and this is in good agreement with the fact that only IBA transport is affected in the *rib1* roots, while IAA transport occurs normally. This result also places *rib1* in a class of IBA resistant mutants distinct from those previously described by Zolman et al. (2000); to the best of our knowledge, *rib1* is

the only mutant that is resistant to IBA for induction of lateral roots, in addition to being resistant to IBA, NPA, TIBA and HFCA in root elongation assays, while retaining wild-type sensitivity to IAA in all assays.

rib1 also shows resistance to NPA inhibition of hypocotyl and primary root elongation, and to inhibition of lateral root formation by NPA and TIBA. Interestingly, we found that although IAA transport itself is not affected by the *rib1* mutation, its inhibition by NPA is abolished by this mutation for root acropetal transport. The *rcn1* mutant shows a similar phenotype: though root acropetal IAA transport in this mutant is normal, it shows defects in regulation of this transport by NPA (Rashotte et al., 2001). It has therefore been suggested to be an auxin transport regulator. By analogy, RIB1 could be a regulator of IBA transport, directly affecting IBA transport and affecting the response of some flows of IAA to NPA.

Materials and methods

Isolation and preliminary characterization of *rib1* has been described previously (Poupart and Waddell 2000). The *rib1* mutation is in the Nossen ecotype of Arabidopsis; Nossen is used as the wild type control in all assays.

Chemicals

3-[5(n)-^{3}H]-Indole acetic acid (27 and 25 Ci mmol^{-1}) was purchased from Amersham (Arlington Heights, IL) and 3-[^{3}H(G)]-Indole butyric acid (25 Ci mmol^{-1}) was prepared in a custom synthesis under conditions designed to label the indole ring by American Radiolabeled Chemicals (St. Louis, MO). NPA was purchased from Chem Service (West Chester, PA). All other chemicals were purchased from Sigma, unless stated otherwise.

Growth conditions for auxin transport assays in seedlings

Seeds were soaked in distilled water for 30 minutes and surface sterilized with 95% ethanol for 5 minutes and 20% bleach with 0.01% Triton X-100 for 5 minutes. After 5 washes in sterile distilled water, seeds were germinated and grown on 9-cm Petri plates containing sterile control medium containing 0.8% agar (Sigma type M, plant tissue culture), 1X Murashige and Skoog salts, pH 6.0; 1.5% sucrose; 1 μg mL^{-1} thiamine; 1 μg mL^{-1} pyridoxine HCl; 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.

Hypocotyl basipetal transport assay

Hypocotyl transport measurements were made on 5-day-old seedlings grown under low light to elongate the hypocotyl. Seedlings were transferred to control plates and oriented vertically such that the shoot apical meristems were aligned. In this assay, mixtures containing 1% agar, 100 nM ^{3}H-IAA or ^{3}H-IBA with either 100 μM NPA or DMSO at the same concentration (1%) were prepared in 3 ml scintillation vials. A narrow stem transfer pipette was carefully inserted into the hardened agar mixture to produce a 1 mm diameter cylinder of agar. This cylinder containing radioactive auxin mixture was applied such that the agar just touched the tip of the hypocotyl from which the shoot apical meristem and cotyledons were cut. Plates remained vertically oriented in the dark, to avoid auxin degradation by light (Stasinopoulos and Hangarter, 1990). Radioactive auxin transport was measured after 5 h by scintillation counting of a 5 mm segment of the base of the hypocotyl. In Table 4.1, the average and standard error of the mean of 30 individuals from 3 assays are reported.

Root transport assays

Basic root auxin transport measurements were made on 6 or 7-day-old vertically grown seedlings as in Rashotte et al., 2001. 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. Standard placement of radioactive agar lines was at the root tips for root basipetal transport (RBT) and just on the root side of the root shoot junction for root acropetal transport (RAT). In each of these assays, mixtures containing 1% agar, 100 nM ^{3}H-IAA or ^{3}H-IBA with either 100 μM NPA, TIBA or 1% DMSO were prepared in 3 ml scintillation vials and prepared and applied as above. Auxin transport was measured after 5 h for RBT by first removing the 1 mm of tissue in contact with the agar line, then cutting a 5 mm segment from the site of application along the desired length. In RAT measurements were made after 18 hr from an application site at the root shoot junction using a 5 mm segment at the root tip. Segments were measured as above.

In Table 4.1, the average and standard error of the mean of 59-80 individuals from 5-7 assays are reported.

Inflorescence auxin transport assays

Plants for inflorescence assays were grown on a 1:1:1 mixture of perlite, vermiculite, and Sunshine mix #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 X Hoagland's solution. Light intensity was approximately 90 μmol m^{-2} s^{-1}.

Inflorescence transport measurements were conducted on approximately 25-day-old plants as described previously (Okada et al., 1991, Brown et al., 2001). Care was taken to insure WT and *rib1* inflorescence stems of matched length were used. In this assay 100 nM ^{3}H-IAA or ^{3}H-IBA were applied to a 20 mm inflorescence segment and transport into the basal 5 mm of that inflorescence segment was measured after 18 hours. Each segment was placed into 2.5 ml of scintillation fluid and the amount of radioactivity within each sample was determined using a Beckman LS6500 Scintillation counter for 2 min. As *rib1* segments tend to be more slender than WT segments, values were corrected for this weight difference. The corrected values were obtained as follows for wild-type: counts for wild-type were divided by the average weight of wild-type segments (= 12.2 mg), to obtain counts/weight, and this value was multiplied by the average weight of wild-type and *rib1* segments (= 9.99 mg), to obtain counts per average weight segments. For *rib1* segments, the values were calculated in a similar fashion: [(counts for *rib1*) / (average weight of *rib1* segments 7.7777 mg)] X (average weight of wild-type and *rib1* segments = 9.99 mg). In Table 4.1, the average and standard error of the mean of 14-15 individuals from 2 or 3 assays are reported.

Hypocotyl length determination

Hypocotyl length was determined by growing seedlings on sucrose-free growth medium (GM) solidified with 0.7% Difco agar (Table 4.4) or on GM with 1.5% sucrose,

0.8% Noble agar (Table 4.3). GM consists of 1X MS basal salts, 1% sucrose, 0.5 g/l MES (2-[N-Morpholino]-ethanesulfonic acid), 1 mg thiamine, 0.5 mg L^{-1} pyridoxin, 0.5 mg L^{-1} nicotinic acid, 100 mg L^{-1} myo-inositol, with pH adjusted to 5.7 with 1 N KOH (Valvekens et al., 1988). Seedlings were germinated and grown in continuous dark, high white light (approx. 90 μmoles m^{-2} s^{-1}), low white light (approx. 5 μmoles m^{-2} s^{-1}), red light or blue light. White light was provided by cool white (Sylvania) fluorescent tubes; red light was provided by F40 gold (Sylvania) fluorescent tubes filtered by Rohm and Haas red Plexiglas No. 2423 (3.18 mm thick, Cadillac Plastic), and blue light was provided by F40 Blue (Sylvania) fluorescent tubes filtered by Rohm and Haas blue Plexiglas No. 2424 (3.18 mm thick, Plastiques Marcon). The far-red light experiments were done in the laboratory of Professor X.-W. Deng at Yale University. Low white light conditions were achieved by placing plates away from the light source and plates received mostly indirect light in this case. Hypocotyl length was determined on seven-day-old seedlings grown in the absence of sucrose or on five-day-old seedlings grown on 1.5% sucrose. Hypocotyls were measured either directly with a ruler or by tracing magnified seedlings using an overhead projector. The tracings were then digitally scanned, and measured using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/). Data for hypocotyl elongation in the absence of sucrose from one representative assay is presented in Table 4.4. Similar results were obtained in one to 5 other trials. Data presented for hypocotyl elongation with sucrose is the average of 7 or 9 trials, as indicated.

Effects of IAA and IBA on hypocotyl elongation

Seeds were surface sterilized by vapor phase sterilization (Clough and Bent, 1998), and then stratified four to seven days in the dark at 4°C before being germinated. IAA and IBA were dissolved in 1N NaOH and diluted in water to a final stock concentration of 1 mg mL^{-1} and filter sterilized. Appropriate amounts of the sterile stocks were added to media after autoclaving to obtain the different concentrations required.

Hypocotyl elongation assays were performed on horizontally oriented GM plates containing 0.8% (w/v) Difco agar. 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}). Wild-type and *rib1* seedlings were plated to each of the two halves of the same plate, to ensure that they were being exposed to exactly the same conditions. Hypocotyl length was determined on five day-old seedlings by tracing magnified seedlings (approx. 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 NIH Image program. Similar results were obtained in three separate trials for each light condition. Data from a single representative trial are presented.

Lateral root formation

Lateral root formation assays were performed as described in Rashotte et al., 2001, with some modifications. Seedlings were germinated on vertically oriented plates containing GM with 1.5% sucrose and 0.8% Noble agar under low white light conditions (5 μmol m^{-2} s^{-1}). After 4 days, seedlings were transferred to the same media containing the indicated amounts of auxins or transport inhibitor. The number of lateral roots on the whole primary root was counted under a dissecting microscope after 6 more days of growth. Each value is the average for 17 or 18 individual seedlings. Similar results were obtained in three separate trials. In Figure 4.3, data from a single representative trial are presented for IAA and IBA. Figure 4.5 shows combined data from 2 (NPA) or 3 (TIBA) trials.

For all hypocotyl elongation and lateral root formation assays, wild-type and mutant seedlings were placed on two halves of the same plate to insure exposure to identical conditions.

Effect of NPA on seedling growth

Sterile and stratified wild-type and *rib1* seeds were plated on a horizontal line across on two halves of a Petri dish containing GM with 1% sucrose and 0.1μM NPA, and grown under high white light for 14 days. A picture was taken on a Leica stereomicroscope at a magnification of 3.15X.

Statistics

Statistical analyses of data were performed using Microsoft Excel. The data were analyzed by two-tailed Student's t-tests for equal variance when comparing wild-type and *rib1* with no prior assumption to this data. The *P* values are reported.

Acknowledgements

We wish to thank: Dr. X.-W. Deng (Yale U.) for use of his facilities for far-red experiments and R. Fry for help with performing these experiments; Dr. M.L. Tierney (U. of Vermont) for help with initial hypocotyl elongation experiments and use of her facilities, and Marianne Marcoux (Waddell laboratory) for technical help.

Chapter 5: Summary and final conclusion

Overview of results presented

When the work presented in this thesis was first initiated, I, like many other plant biologists, had little awareness of the role of auxins other than indole-3-acetic acid (IAA) in plants, and though I knew of their existence, I did not know whether they occurred at physiologically relevant levels in plants. The characterization of the hormone response profile of the *rib1* mutant, which was initially identified as a mutant defective in root gravitropic response, showed it was resistant to IBA and 2,4-D, but not to IAA and NAA in root elongation assays. When we discovered this, we were unaware of other mutants with similar phenotypes, and some textbooks still (wrongly) referred to IBA as a synthetic auxin. The occurrence of such an IBA specific mutant suggested IAA and IBA response were genetically separable, and characterization of the *rib1* mutant could help define the role of IBA in plants. As detailed in the general introduction of Chapter 1, several other IBA specific mutants have since been discovered and/or characterized, and several previously characterized mutants have been shown to be specifically defective in IBA response, such as ethylene signaling and light response mutants. Since physiological studies aimed at studying the differential effects of IAA and IBA are sometimes complicated by the fact that these two hormones can be interconverted, analysis of the phenotypes of IBA specific mutants has helped to genetically dissect the role of these two endogenous auxins.

Phenotypic analysis revealed that *rib1* seedlings presented a shorter primary root, increased lateral root formation, and a longer hypocotyl in certain light and media conditions, but adult *rib1* plants were undistinguishable from wild-type. These results imply IBA could be important in defining seedling morphology. Others assays showed *rib1* wasn't resistant to other classes of plant hormones, but that it did have an altered response to the IAA transport inhibitors NPA, TIBA and HFCA. Such hormone and inhibitor response phenotypes are characteristic of auxin transport mutants, so this suggested RIB1 affected auxin transport in some way. We therefore established a collaboration with the laboratory of Dr. Muday, who specializes in the study of auxin transport in plants, to investigate this possibility further.

The transport of IBA in seedlings had not been previously characterized, and some contradictory results on transport of IBA in inflorescence stems had been published (one study reported primarily acropetal transport of IBA in Arabidopsis inflorescence stems, while most other studies, in other species, reported primarily basipetal (polar) transport of IBA in stems). Our analysis of IBA transport in Arabidopsis demonstrated that IBA transport in seedlings is polar and occurs in three distinct flows: from the apical meristem to the base of the hypocotyl (hypocotyl basipetal transport), from the base of the root to the root tip (root acropetal transport) and from the root tip toward the base over a short distance (root basipetal transport). This mirrors exactly the three flows of IAA transport described in Arabidopsis and other seedlings, but dissimilarities of transport between the two endogenous auxins were also noted. First, the amounts of radiolabelled IBA transported were 2 to 4 fold higher than those of IAA. Whether this reflects higher transport capacity or simply higher uptake of IBA from the medium remains to be experimentally addressed. Second, the IAA transport inhibitors NPA and TIBA did not inhibit IBA transport. Third, the *eir1* and *aux1* mutants, that affect IAA transport, do not affect IBA transport in seedlings. Finally, we found no evidence for polar transport of IBA in inflorescence stems of Arabidopsis, even after long incubation periods and using high auxin concentrations. Taken together, our results suggest the existence of a pathway for IBA transport that is distinct from that of IAA transport, as the transport of these two auxins is differently regulated. We also found evidence for differential response of hypocotyls to IAA and IBA. IBA stimulates hypocotyl elongation under high light conditions on rich medium, while we, and others, did not see such an effect with IAA. At high concentrations of auxin, and in low light or dark conditions, both auxins inhibit hypocotyl elongation. We also confirmed that light affected the transport of IAA, and showed it also affected IBA transport in a similar manner in hypocotyls. This study is the first detailed characterization of IBA movement in seedlings, and served as groundwork to investigate auxin transport in the *rib1* mutant background.

Analysis of auxin transport in *rib1* (Chapter 4) revealed that all flows of IBA transport were affected by this mutation, while IAA transport was unaffected. Moreover, we were able to correlate modifications in IBA transport with phenotypic alterations in *rib1* and with the effects of exogenous application of IBA. Recent studies of IAA

transport have shown that root acropetal and root basipetal transport of this auxin are important for lateral root production and gravitropic response, respectively. Our studies suggest IBA transport flows could have similar functions. Root acropetal IBA transport was increased almost two fold in *rib1*, and lateral root formation was similarly increased in *rib1*; this correlates well with the fact that IBA can stimulate branching of wild-type roots. On the other hand, root basipetal transport of IBA was reduced, and so was gravitropic response of this mutant. In hypocotyls, IBA transport is also reduced in *rib1* relative to wild-type; exogenous IBA inhibits hypocotyl elongation, and accordingly, there is increased hypocotyl elongation in *rib1* in the same conditions. Our results therefore support a role for IBA transport in regulating seedling development that is at least partially overlapping with that of IAA.

Possible identity of the RIB1 protein

The RIB1 locus has not been cloned; therefore we cannot determine homology of RIB1 to previously cloned proteins. However, our results allow us to speculate on RIB1 protein identity, as *rib1* mutants present many phenotypes in common with characterized and cloned mutations. Notably, auxin and transport inhibitor resistant phenotypes are seen in IAA transport mutants, and *rib1* mutants were shown to be specifically defective in IBA transport. Our results are therefore most consistent with RIB1 encoding either an IBA specific influx or efflux transporter, which could be a member of the AUX, PIN or ABC transporter families, or a transport regulator, such as the kinase and phosphatase components RCN1 or PID1. Alternatively *rib1* could affect IBA transport indirectly. We therefore cannot rule out other functions for the RIB1 protein until this issue is resolved through cloning of the gene.

Probable role of the endogenous auxin IBA

Our analyses of RIB1 function and of the effects of IBA on plant growth and development compared to that of IAA suggest IBA is a physiologically important auxin specifically affecting seedling development. As was suggested by previous

investigations, IBA affects root formation, but our analyses have also revealed a role for IBA in regulating light response, notably in regulation of hypocotyl elongation. As mutants defective in peroxisomal function and IBA to IAA conversion present auxin related phenotypes, IBA acts at least partially through its conversion to IAA. Results presented here, in addition to results of analysis of other mutants presented in the introduction suggest however that IBA also acts as an auxin independently from its conversion to IAA.

Future prospects:

Determination of exact levels of IBA in different tissues of wild-type and *rib1* would also be of interest to confirm that the modifications of transport of tracer amounts of IBA that we have demonstrated correspond to modifications in endogenous IBA distribution in this mutant. As some of our results (notably resistance of *rib1* to IAA transport inhibitors, and modification of sensitivity of IAA transport to these same inhibitors in *rib1*) suggest there could be crosstalk between IAA and IBA transport pathways, it would also be interesting to look at IBA transport in other so-called IAA transport mutants. Competition studies of IAA and IBA transport in different tissues could help define the extent of crosstalk between the two transport pathways.

As detailed in Appendix I, double mutant analysis has also begun to reveal interesting interactions between *rib1* and previously characterized mutants, thereby defining response pathways that depend on IBA or RIB1 for proper functioning.

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Appendix I: Double mutant analysis

Abstract:

Characterization of phenotypes and of auxin transport in *rib1* suggests this mutant is specifically affected in IBA transport. As this is the first mutant demonstrated to have such a phenotype, we wanted to determine if RIB1 interacts with proteins encoded by loci defined by previously characterized mutations. *rib1* mutants were crossed to mutants affected in auxin response (*axr1* and *axr2*), auxin (IAA) transport (*aux1, axr4, eir1, tir2*), regulation of IAA levels (*rty1*) or with defects in light response (*hy5*). In most cases, the response of double mutants to hormones and inhibitor was studied, as well as hypocotyl elongation in different light conditions. Auxin response (*axr1*) and transport (*aux1, axr4*) mutants could partially or completely suppress the long hypocotyl conferred by *rib1*, indicating this phenotype depends on proper auxin transport and response. On the other hand, the *aux1*, *axr1* and *axr4* mutations had additive effects with *rib1* for 2,4-D resistance in root elongation assays. Preliminary analysis showed that *axr2 rib1* double mutants had hyponastic cotyledons, a novel phenotype not seen in either single mutant, that might reflect reduced auxin response in this tissue. *rib1* and *hy5* interact in a synergistic manner for auxin resistance, and present additional novel phenotypes not seen in either single mutant: reduced plant height, loss of apical dominance, reduced fertility and occurrence of rare seedlings lacking basal structures (roots and hypocotyls). Results presented here support a role for HY5 in an auxin dependent or responsive pathway in which RIB1 would be involved. The auxin transport mutant *eir1* can potentiate the 2,4-D resistance of *rib1*, even though the *eir1* single mutant is slightly more sensitive to this auxin than wild-type. *eir1 rib1* double mutants are also less resistant to ACC than *eir1* single mutants, indicating *rib1* can partially suppress *eir1* ACC resistance. *tir2 rib1* double mutants had reduced fertility caused by reduced shedding of pollen by anthers. As this mutant is also suggested to affect auxin transport, these results are consistent with a model in which *rib1* acts through its effects on auxin transport. Finally, we found *rib1* could partially suppress the excessive root proliferation resulting in inhibition of development in the auxin overproducing *rty1* mutant. Overall, our results suggest extensive cross-talk between IAA and IBA response and transport pathways.

Introduction:

In order to better understand the role of RIB1 and to attempt to define the response pathway(s) in which this protein could be involved, double mutant analysis was performed. We crossed *rib1* to mutants in IAA response (*axr1* and *axr2*), IAA transport (*aux1, axr4, eir1, tir2*), regulation of IAA levels (*rty1*) and light response (*hy5*). These mutants are described, and the function of the affected genes is discussed, in the general introduction (Chapter 1), but will also be reviewed here, with emphasis on mutant phenotypes. Please also refer to the appropriate section in Chapter 1 for more details and to Tables 1.2 and 1.3 for complete references to phenotypic analysis. In this appendix, we present the preliminary analyses of these double mutants, and suggest further experiments that could lead to a better understanding of the function of the genes involved in the interactions uncovered by this work.

Auxin response mutants:

axr1 and *axr2* were both isolated based on auxin resistant root elongation. The *axr1* mutations (including the *axr1-3* allele used in this study) have pleiotropic effects: they affect root and shoot elongation, root and hypocotyl gravitropic response, apical dominance and hypocotyl elongation in the dark (see Table 1.3 for more details and references). Mutations at the *AXR1* locus reduce the response to the auxins 2,4-D, IAA, IBA and NAA, but also to the hormones benzyladenine (BA – a cytokinin), ethylene, methyl jasmonate, epibrassinolide and ABA (Table 1.3).

As stated in Chapter 1, AXR1 is involved in regulated proteolysis through the ubiquitin/proteasome pathway (see overview in Figure 1.6C). Specifically, *AXR1* encodes one half of a bipartite E1 like enzyme involved in RUB modification of the SCF^{TIR1} E3 complex (step 3a of Figure 1.6). AXR1 protein function is necessary for RUB modification of the cullin subunit of SCF^{TIR1} (del Pozo and Estelle, 1999), but has also been suggested to be important for the function of other SCF type E3 enzymes, such as those involved in jasmonic acid, cold and light signaling (Schwechheimer et al., 2002). This can explain, at least in part, the pleiotropic nature of *axr1* mutations.

axr2-1 is a dominant mutation resulting in very small plants, with agravitropic roots, hypocotyls and inflorescences, and short roots and hypocotyls in a variety of light conditions (see details and references in Table 1.3). Interestingly, *axr2-1* is more sensitive to auxins (2,4-D, IAA and IBA) for induction of lateral roots. As auxins have very little effect on hypocotyl elongation in *axr2-1*, it has been suggested AXR2 is more important for auxin response in hypocotyls than in roots.

AXR2 encodes the IAA7 protein, which is a member of the Aux/IAA family of proteins. As discussed in Chapter 1, Aux/IAA proteins are short-lived proteins involved in regulation (together with the ARF family of proteins) of the many genes involved in auxin response. The *axr2-1* mutation affects domain II of IAA7, a destabilizing domain that maintains the short half-life of the wild-type protein. The mutant AXR2 protein is therefore stabilized, and this mutation is dominant.

Auxin transport mutants

aux1-7 root elongation is resistant to inhibition by IAA, IBA, 2,4-D, ethylene and the cytokinin benzyladenine. *aux1-7* is, however, not resistant to IAA and IBA for lateral root production. This mutant also has a short hypocotyl in the dark, less lateral roots and reduced root gravitropic response (see Table 1.2 for more details of phenotypes and references). *AUX1* encodes a protein with similarity to amino acid permeases suggested to function as an auxin influx carrier. Consistent with this hypothesis is the fact *aux1* mutants are not resistant to NAA and that this auxin can restore lateral root formation and gravitropic response in mutants. NAA influx into cells has been shown not to be saturable, indicating this auxin probably enters cells passively without the help of a carrier (Delbarre et al., 1996). *aux1-7* results in modified IAA accumulation patterns in leaves and roots, which is also consistent with a defect in auxin transport in this mutant.

axr4 is resistant to IAA, 2,4-D, NPA and TIBA in root elongation assays, and has defects in root gravitropism, slightly longer roots and produces a reduced number of lateral roots. *axr4* is not resistant to NAA, and NAA can restore gravitropism in this mutant, leading to the suggestions *axr4*, like *aux1*, is affected in auxin influx. The AXR4 protein has not been cloned.

The *eir1* mutant was isolated based on ethylene insensitive root growth. Roots of this mutant are agravitropic, longer and resistant to inhibition by TIBA. Other allelic mutations show reduced efflux of preloaded IAA. These mutations were shown to lie in the *PIN2* gene, which is a member of a family of membrane proteins suggested to act as auxin efflux carriers. *In situ* hybridization and immunolocalization studies show that *PIN2* is expressed in root tips, specifically in the cortical and epidermal cell layers (Müller et al., 1998).

tir2 is the least well-characterized of the mutations used to construct double mutants. It was isolated by NPA resistant root growth and has defects in hypocotyl elongation and lateral root formation, but no phenotypic characterization of this mutant has been published, and the affected gene has not been cloned.

The *rty* mutant has increased auxin accumulation

Phenotypes of *rty* mutants include a short hypocotyl and absence of apical hook formation in the dark, dramatic increases in lateral, adventitious and ectopic root formation, epinastic cotyledons and infertility due to lack of inflorescence production (Boerjan et al., 1995; Celenza et al., 1995; Gopalraj et al., 1996; King et al., 1995; Lehman et al., 1996; Windsor 2001). These phenotypes can be phenocopied by growth of wild-type plants on media containing high concentrations of auxin, and increased IAA levels have been measured in the mutants (Celenza et al 1996; King et al., 1995). The *rty1-5* allele used in this study was isolated in the Waddell lab and is a strong allele that does not normally produce true leaves but rather produces multiple adventitious and ectopic roots instead of developing normally (Windsor, 2001). RTY encodes a protein with similarity to tryptophan aminotransferases (Gopalraj et al., 1996), possibly involved in IAA biosynthesis from tryptophan, or in regulating IAA levels (Bartel, 1997).

The *hy5* mutant

hy5 displays a long hypocotyl phenotype in red, far-red and blue light (Koornneef et al., 1980), but also displays phenotypes reminiscent of those seen in auxin resistant

mutants, including defects in primary and lateral root gravitropism, increased number of lateral root primordia and increased lateral root elongation (Oyama et al., 1997). In addition, *hy5* mutants have decreased secondary thickening of roots and hypocotyls, and reduced greening of hypocotyls and roots in the light when compared to wild-type (Oyama et al., 1997). The *HY5* gene was cloned and the carboxy-terminal end of the protein is homologous to the DNA binding and dimerization domain of bZIP proteins (Oyama et al., 1997). It can bind and activate transcription from the promoter of light regulated genes, including *CHALCONE SYNTHASE* (*CHS*) (Ang et al., 1998). HY5 is hypothesized to be a positive regulator of photomorphogenesis whose levels and activity are regulated by light (Osterlund et al., 2000) and by interaction with other proteins, notably the HY5 homolog HYH, and members of the COP/DET/FUS family of proteins. Specifically, HY5 directly interacts with the COP1 protein, and this light dependent interaction results in negative regulation of HY5 (Ang et al., 1998).

Results

Hypocotyl elongation in double mutants

Results of hypocotyl elongation assays in red light are illustrated in Figure AI.1. Under these light conditions, *rib1* seedlings have significantly longer hypocotyls than the wild-type background ecotype No-0 (Student's t-test *P* values: 2.4×10^{-11} to 6.5×10^{-7} for the assays presented). In contrast, the auxin influx mutant *aux1-7* hypocotyls are not significantly different from their wild-type background (Col-0) in these conditions (Panel A). Though *aux1* itself did not have short hypocotyls, this mutation was able to partially suppress the long hypocotyl phenotype caused by *rib1*: double mutants had hypocotyls of intermediate length, slightly yet significantly longer than *aux1* hypocotyls (one-tailed t-test *P* value 0.027). The results of similar assays done with *axr4-2*, which has also been suggested to affect auxin influx, are different (Panel B). Under the conditions of the assays, *axr4* hypocotyls were significantly shorter than wild-type (t-test *P* value 0.004), however this mutation did not suppress the long hypocotyl phenotype caused by *rib1*, as *rib1* and *axr4 rib1* hypocotyl lengths were not significantly different. Interestingly, in white light and in the same media conditions, *axr4 rib1* double mutant hypocotyls were intermediate between *rib1* and *axr4* length, indicating *axr4* can suppress *rib1* induced hypocotyl elongation in different light conditions (hypocotyl lengths: *axr4*: 1.66 mm, *rib1*: 3.24 mm; *axr4 rib1* double mutants: 2.36 mm).

The results of hypocotyl elongation assays for the auxin response mutant *axr1-3* is presented in panel C of Figure AI.1. In red light, *axr1-3* hypocotyls are shorter than wild-type Col-0 (t-test *P* value 2.34×10^{-18}), and *axr1* completely suppresses the long hypocotyl phenotype caused by *rib1*, as the double mutant hypocotyls are not significantly different from *axr1* single mutant hypocotyls.

Results of hypocotyl elongation assays for other double mutants are not presented in Figure AI.1. As EIR1 is specifically expressed in roots, we did not investigate hypocotyl elongation in red light in the *eir1 rib1* double mutants. The identification of *hy5 rib1* double mutants was not made early enough to allow us to analyze hypocotyl elongation in the absence of hormone in these double mutants. Preliminary analysis

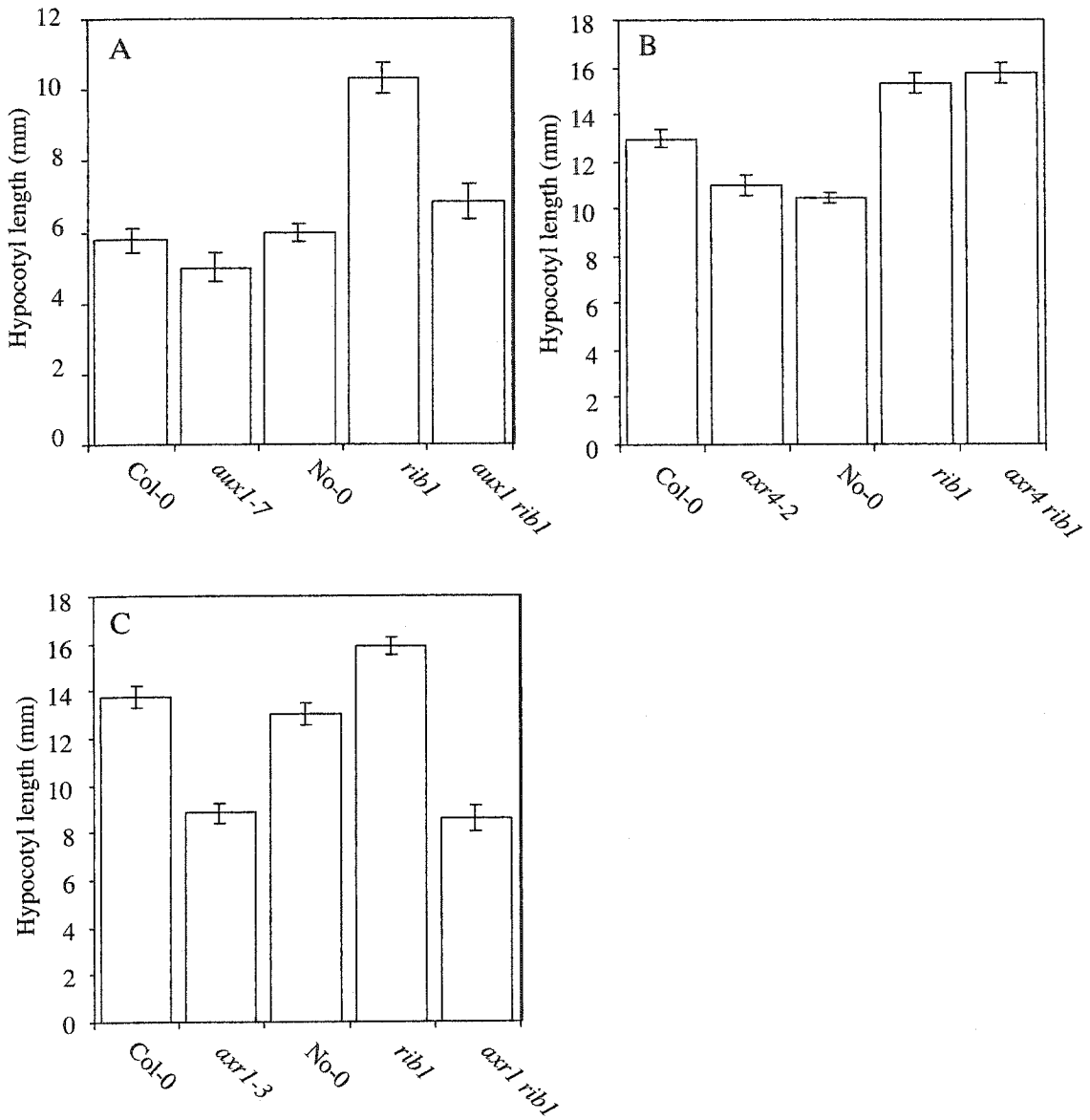

Figure AI.1: Hypocotyl length of 7 day-old seedlings grown in red light. A) *aux1-7, rib1,* their respective wild-type backgrounds (Col-0 and No-0) and *aux1 rib1* double mutants are compared. B) *axr4-2, rib1*, their respective wild-type backgrounds (Col-0 and No-0) and *axr4 rib1* double mutants are compared. C) *axr1-3, rib1,* their respective wild-type backgrounds (Col-0 and No-0) and *axr1 rib1* double mutants are compared.

shows *hy5 rib1* double mutant hypocotyls are much longer than either parent on media containing 2,4-D (about 2 fold longer than either *hy5* or *rib1* single mutant hypocotyls, data not shown). Unfortunately, we did not unambiguously identify homozygous *rib1 axr2* double mutant lines, and this mutant combination could therefore not be included in the analysis of hypocotyl elongation presented here (see below).

Hypocotyl elongation in *rty1 rib1* double mutants was not measured, but can be seen in Figure AI.2. *rty1-5* displays a short hypocotyl (panel A). In addition, this mutant presents strongly epinastic cotyledons and true leaves, a short primary root and excessive production of adventitious and ectopic roots on the whole seedling, eventually resulting in production of a mass of root-like tissue that cannot develop to produce more than the first two true leaves because of a loss in specification of the apical meristem. *rty1-5* is a strong allele and homozygotes never produce inflorescences or seeds. In the analysis of the F2 generation following a cross between *rib1* and *rty1-5*, both 2,4-D resistance and *rty* individuals segregated, as expected. Careful examination of F2 individuals revealed the existence of different classes of *rtys*: individuals with weak (panel B), intermediate (panel D) and strong (not shown – similar to panel A) *rty* phenotypes were recovered. Representatives from all *rty* classes were transferred to soil, but only individuals with weak phenotypes survived and produced flowers. A single individual produced approximately ten seeds, which were sown on the plate shown in panel C. As can be seen, all the seeds that germinated on this plate had a rooty phenotype, indicating the parent was homozygous for *rooty*. We conclude that *rib1* partially suppressed the short hypocotyl, reduced adventitious and ectopic root production, and partially alleviated the inhibition of root elongation caused by *rty1-5,* thereby allowing development of the plant to the point of seed production.

Hormone and inhibitor resistance of double mutants

As a first step to investigate auxin resistance in double mutants, seeds of single mutants, wild-type and double mutants were plated on 2,4-D containing medium, and root lengths were measured after 7 days of growth. As expected based on published reports *rib1* and the *aux1*, *axr1*, and *axr4* mutants were shown to be 2,4-D resistant, as evidenced

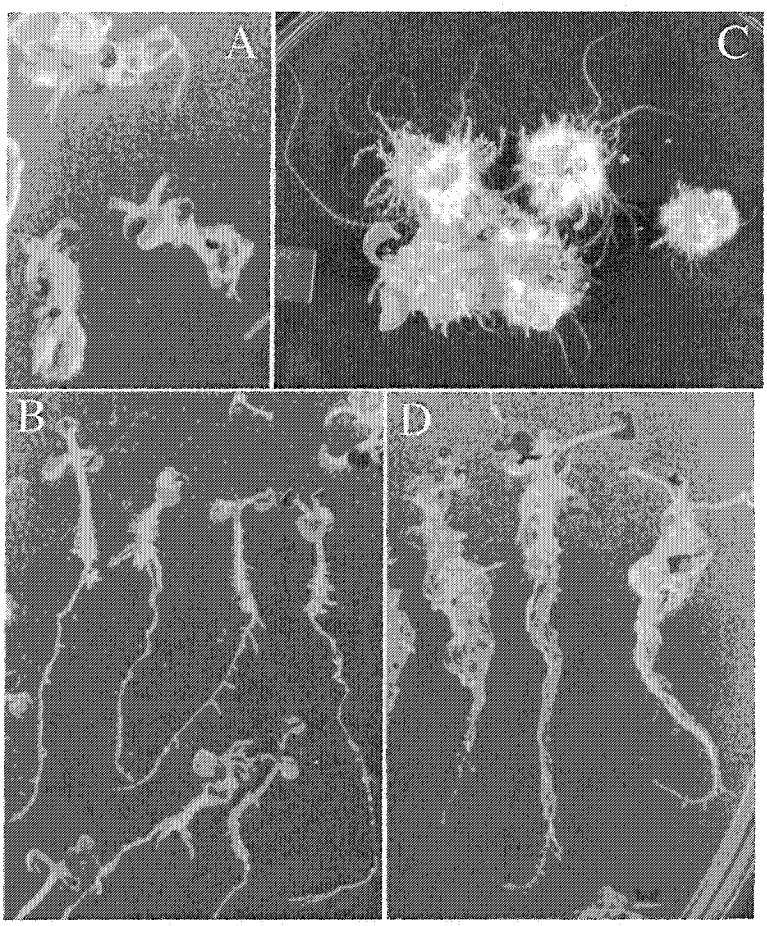

Figure AI.2 Pictures of 14 d old *rty1-5* homozygous seedlings (A) compared to *rty1 rib1* individuals recovered in the F2 generation, showing weak (B) and intermediate (D) Rty phenotypes. Weak rtys such as the ones shown in panel B were transfered to soil, and one individual produced an inflorescence and, eventually, 10 seeds. These 10 seeds were sown on the plate seen in C, which was photographed after approximately 3 weeks of growth (only 7 of the 10 seeds germinated).

by the fact their roots were significantly longer than wild-type on media containing this auxin (see Figure AI.3 A-C). *aux1 rib1*, *axr1 rib1* and *axr4 rib1* double mutant roots were significantly longer than the corresponding single mutants (one-tailed t-test *P* values between 4.3×10^{-14} and 0.04 for the data presented in Figure AI.3 A-C), indicating an additive interaction.

A surprising result was obtained when we investigated 2,4-D resistance of *eir1 rib1* double mutants (Figure AI.3D). As previously reported, *eir1* is slightly, yet significantly more sensitive to 2,4-D than wild-type at certain concentrations. The double mutant, unexpectedly, was much more resistant to 2,4-D than the *rib1* single mutant (t-test *P* value 2.2×10^{-11}), indicating the *eir1* mutation can potentiate the 2,4-D resistance conferred by *rib1*.

Two families of *hy5 rib1* mutants were analyzed, as wild-type background affects auxin resistance and hypocotyl elongation. The *hy5-1* mutant is in the Landsberg *erecta* background. Under our growth conditions, we found this ecotype to be more resistant than wild-type Nossen and Columbia to auxins, and to have a longer hypocotyl, making analysis of double mutant phenotypes somewhat difficult with this line. We therefore also used the *hy5-215* allele, which is in the Columbia background. We found this background to be similar to our standard Nossen wild-type, in terms of hypocotyl length, and response to auxins (data not shown). We analyzed double mutants from both families, and observed some common phenotypes, but also some differences, which could possibly be attributed to allele strength or ecotype differences. In both families, approximately 1 16^{th} (one sixteenth) of progeny of the F1 of a *rib1* X *hy5* cross presented roots much longer than either single mutant on 2,4-D containing plates (see Figures AI.3E and AI.4A). Though both *rib1* and *hy5* show relatively modest 2,4-D resistance compared to that of other mutants such as *aux1*, *axr1*, and *axr2* (which have average root lengths between 31 and 39 mm, compared to only 7 and 13 mm for *rib1* and *hy5-1*, respectively), double mutants showed a very high level of resistance, consistent with a synergistic interaction. This can also be seen in the root length distribution graph presented in Figure AI.4B, in which 3 different *hy5 rib1* double mutant lines are compared to the parental *hy5* and *rib1* single mutants.

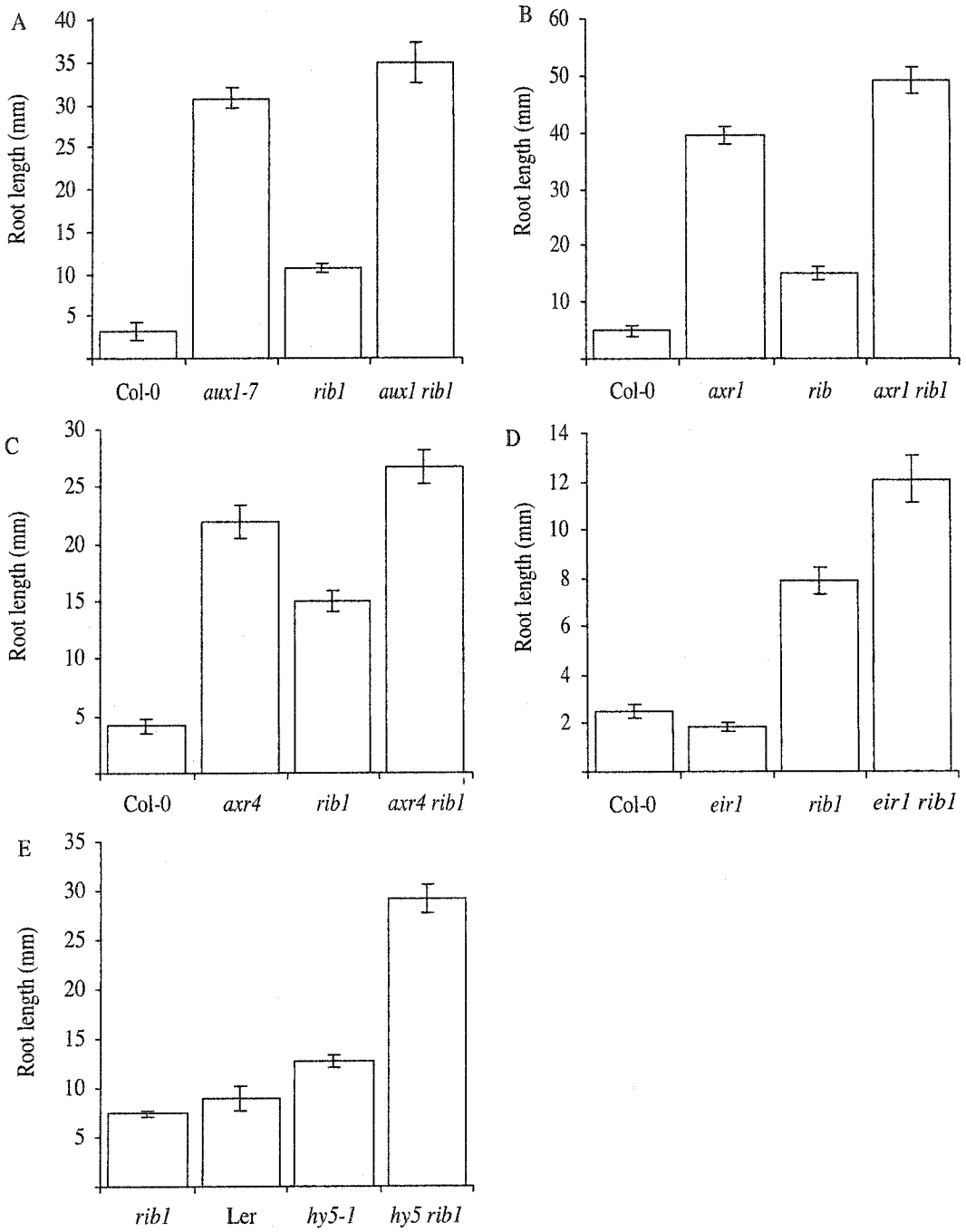

Figure AI.3: 2,4-D resistance of *rib1* double mutants.
Seeds of the indicated genotypes were sown directly on 2,4-D containing media and placed in white light. Primary root length was measured after 7 days of growth. The average of 8 to 55 seedlings is presented, with error bars representing the standard error of the mean.

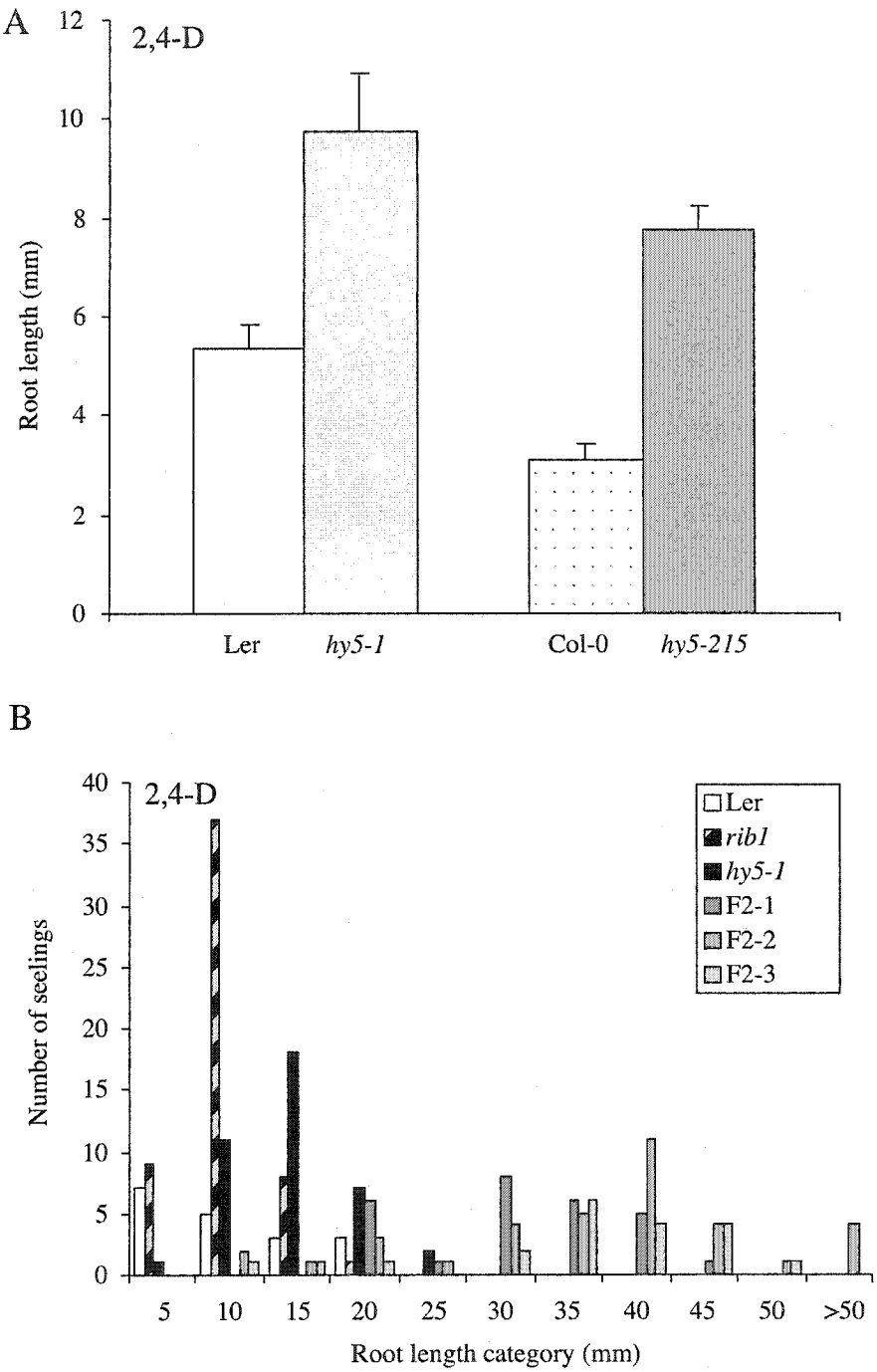

Figure AI.4: Analysis of 2,4-D resistance of *hy5 rib1* double mutants. A: Root length of 7 day-old *hy5-1* and *hy5-215* seedlings on 2,4-D, compared to their appropriate wild-type background ecotype. Averages of 8 to 31 seedlings are presented; error bars represent the standard error of the mean. B: Distribution graph of root length of the progeny of three very resistant F2 individuals, compared to Ler wild-type and single mutants on 2,4-D.

Table AI.1 summarizes the results of root elongation assays done to investigate the response of double mutants to other auxins, hormones and to the IAA transport inhibitor NPA. In most cases, our data is consistent with an additive interaction for the compounds tested. Our preliminary analysis suggests *rib1* may suppress ethylene resistance conferred by *eir1*; root length of the *eir1 rib1* double mutants is intermediate between that of *eir1* (ethylene resistant) and that of *rib1* (normally sensitive to ethylene). In the average of three trials, the root elongation on ACC relative to growth in the absence of hormone is 63% for *eir1*, 27 to 32% for *rib1* and wild-types Col-0 and No-0 and 44% for the *eir1 rib1* double mutant.

Novel phenotypes of double mutants

axr2 rib1 double mutants presented a novel phenotype: the cotyledons of the double mutants were hyponastic, or up-turned, as can be seen in Figure AI.5, panel B. This growth pattern can be compared to that of *axr2* cotyledons, in panel A, which displays cotyledon positioning similar to that of wild-type and *rib1*. Unfortunately we were unable to unambiguously identify a double homozygous *axr2 rib1* mutant, as both mutants confer auxin resistance in a (semi-)dominant fashion, and an insufficient number of lines was analyzed. Further analysis will be required to determine the segregation pattern and inheritance of this phenotype, which was first seen in all the individuals of the F1 generation of a cross between *axr2* and *rib1* homozygotes.

A reduction in seed set was noticed in *hy5-1 rib1* and *hy5-215 rib1* double mutant lines. This was most clearly seen in the F3 generation of the *hy5-1-rib1* cross. When putative *hy5-1 rib1* F2 individuals (seedlings with long roots) were transferred to soil, the resulting adult plants had a novel phenotype: the plants were short with a loss of apical dominance, and looked very bushy due to an increase in the number and branching of inflorescence axes. The average height of double mutant plants was 12.4 cm (compared to 23.3 cm in wild-type under our standard growth conditions), and the number of inflorescences was increased (average of 4.8 inflorescence stems in double mutants, which can be compared to approximately 3.5 in wild-type). Seed set was also much reduced in these plants, with plants producing between 400-500 seeds to no seed at all.

	Hormone tested					
Genotype	IAA	IBA	NAA	ACC / C_2H_4	NPA	ABA
rib1	WT	R	WT	WT	R	S
aux1	R	R	WT	R	n.t.	n.t.
aux1 rib1	+	+	+	+	n.t.	n.t.
axr1	R	R	R	R	R	R
axr1 rib1	+	+	n.t.	+	+	+
axr4	R	n.t.	WT	WT	R	WT
axr4 rib1	+	n.t.	n.t.	n.t.	n.t.	n.t.
eir1	WT - S	WT	WT - S	R	R	WT
eir1 rib1	+	+	+	- *	+	+

Table A1.1: Summary of double mutant analysis: Root elongation assays for hormone and inhibitor resistance.

For single mutants, published results and/or our results are reported. C_2H_4, ethylene (either resistance to the gaseous hormone ethylene or to the ethylene precursor ACC is reported in the literature, our assays were done on ACC); WT, wild-type response; R, more resistant than WT; S, more sensitive than WT; WT –S, wild-type response at most concentrations, but more sensitive at one or two concentrations; n.t. not tested. Double mutants: +, consistent with an additive interaction; - consistent with a suppressive interaction.

* Root length intermediate between *rib1* and *eir1*, indicating *rib1* could partially suppress ACC resistance conferred by *eir1* (see text).

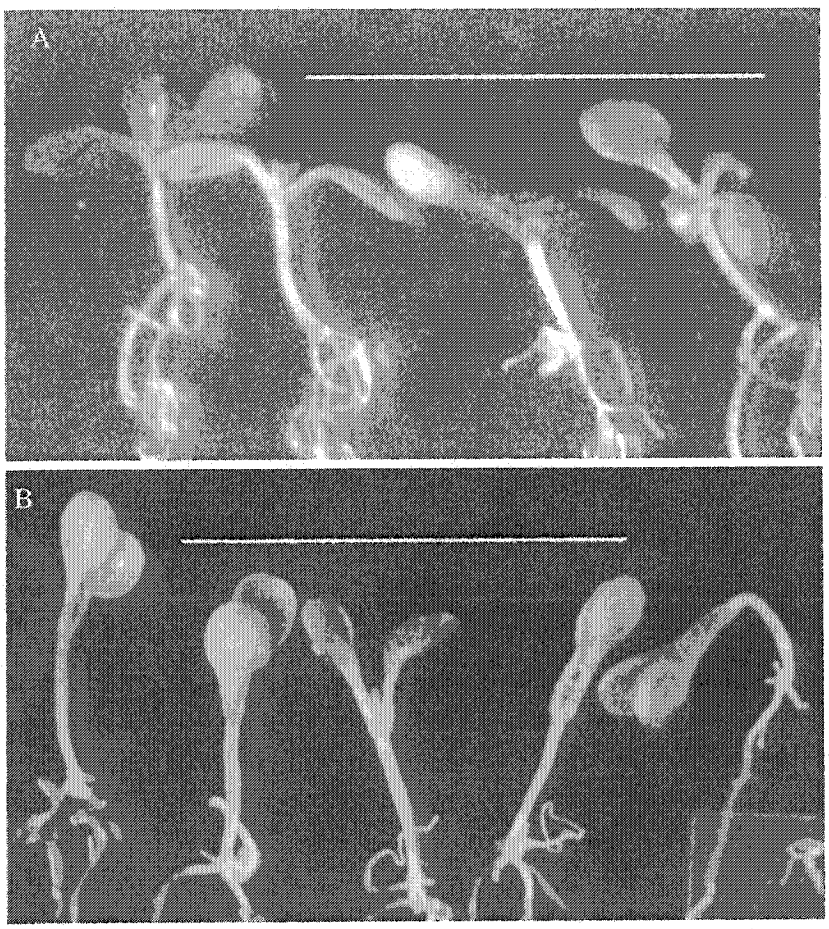

Figure AI.5: Picture of *axr2* (A) and *axr2 rib1* (B) double heterozygous mutant seedlings grown for 7 days on media lacking hormones. Note hyponastic cotyledons of double mutants in B. Scale bar = 5 mm.

Of the seeds produced by these F3 individuals, most did not germinate, and of the seeds that did germinate, most were seedling lethals that died by day 10 after germination. Many of these seedlings also had short roots.

Similar results were obtained following analysis of the progeny of a number of *hy5-215 X rib1* crosses: there was a reduction in seed set in most F2 plants that had very long roots. Analysis of the growth of the F3 should reveal whether the plants harbouring the *hy5-215* allele also show a bushy phenotype, and results in loss of viability in subsequent generations.

Finally, though the majority of *hy5-1 rib1* putative double homozygous mutant seedlings appeared relatively normal despite their increased hypocotyl and root elongation, a few individuals presented novel phenotypes such as those presented in Figure AI.6. In this Figure, wild-type Ler (panel A), *rib1* (panel B) and *hy5-1* (panel C) seedlings grown on 2,4-D can be compared to seedlings, found in the progeny of the very resistant F2 individuals, (panels D through G) that are very small and lack embryonic root, and some or all of the hypocotyl. We still have to determine whether such phenotypes will occur in the F3 in the *hy5-215* families, and segregation of this phenotype is still unclear at present. Loss of fertility of putative homozygous *hy5 rib1* double mutants also complicates these studies, because we have not determined whether loss of individuals could have a relation to defects in embryogenesis.

tir2 rib1 putative double mutant lines showed no difference from either single mutant in seedlings or in adult plants. After seed set however, we noticed many siliques did not fill properly, and upon opening such siliques, we noted many of these contained no seeds (see Figure AI.7). Careful examination of flowers revealed the probable cause of this defect: many *tir2 rib1* double mutant flowers fail to shed pollen (compare flower in panel B of Figure AI.7 to wild-type flower in panel A). Cross-pollination of these male sterile flowers with pollen from a wild-type flower resulted in normal silique elongation and filling, indicating the flowers remain female fertile. No reduction in fertility was associated with either *rib1* or *tir2* alone, so the reduction in fertility of double mutants is a novel phenotype.

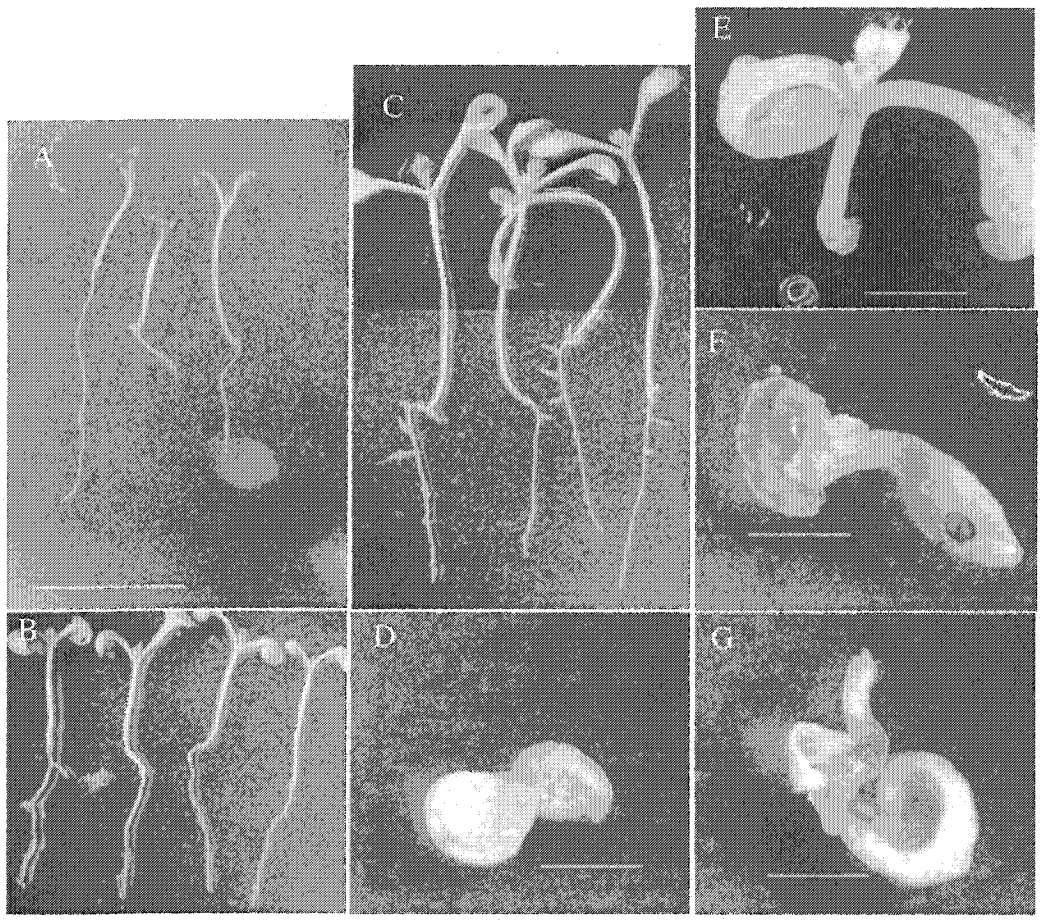

Figure AI.6: Picture of 7 day-old seedlings grown on 2,4-D. A: wild-type seedlings. Note to the left a rare wild-type individual that had a long root under these conditions. B: *rib1* seedlings. C: *hy5-215* seedlings; D through G: Seedlings with abnormal morphology found in the progeny of very resistant F2 individuals, probably homozygous double mutants. Scale bar in A is 5 mm, and same magnification was used for panels B and C. Scale bars in D through G are 1 mm.

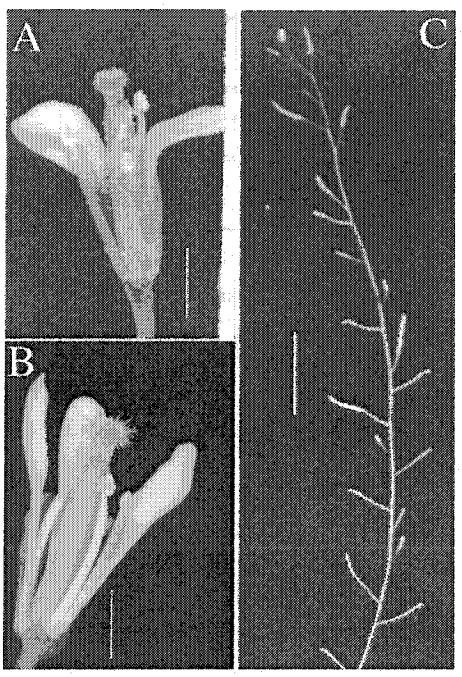

Figure AI.7: Picture of a wild-type flower with visible pollen grains (panel A) compared to a *tir2 rib1* double mutant flower that does not shed pollen (panel B). Scale bar = 1 mm. Panel C: inflorescence of a *tir2 rib1* double mutant with many empty siliques (small), and a few longer siliques with partial seed set. Scale bar = 1 cm

Discussion:

Auxin response and transport mutants affect hypocotyl elongation in *rib1*

The auxin response mutation *axr1* can completely suppress the long hypocotyl phenotype of *rib1*. This suggests the long hypocotyl phenotype of *rib1* depends on proper auxin signaling in hypocotyls. Results presented in Chapter 4 show *rib1* reduces IBA transport in hypocotyls, and IBA can stimulate hypocotyl elongation in wild-type, so an attractive hypothesis is that IBA accumulates in *rib1* hypocotyls, causing increased elongation. When auxin signaling is reduced by the *axr1* mutations, the stimulatory effect of IBA would be abolished. IBA levels have not been measured in wild-type or *rib1* seedlings, so such a hypothesis would require confirmation.

It might at first seem surprising that in red light *aux1* hypocotyl length was not different from wild-type, yet *aux1* partially suppresses the long hypocotyl phenotype of *rib1* in these same conditions (Figure AI.1). However, *aux1* does affect hypocotyl elongation in the dark (resulting in short hypocotyls), showing AUX1 is involved in hypocotyl elongation. Based on our data indicating *rib1* hypocotyl elongation depends on media and sucrose conditions (Chapter 4), it would be interesting to see how hypocotyl elongation in *aux1* and other double mutants reacts to light and media modifications.

Although *aux1-7* itself does not affect IBA transport (Chapter 3), the *aux1-7* mutation partially suppresses the long hypocotyl phenotype of *rib1*. This result suggests there could be crosstalk between the IAA and IBA signaling pathways. This was also suggested by the fact that although IAA transport levels are not changed by *rib1*, *rib1* abolishes regulation of IAA transport by NPA in root acropetal transport (Chapter 4). Alternatively, as increased accumulation of IAA was shown to occur in the aerial portions of *aux1* seedlings (Marchant et al, 2002), accumulation of IAA and IBA because of defects in transport of both auxins could result in supra-optimal auxin levels in hypocotyls and therefore hypocotyl inhibition. The fact that *axr4* can suppress *rib1* induced hypocotyl elongation in white (but not red) light could also suggest cross-talk between IAA and IBA auxin transport pathways, and that these interactions might be modulated by light quality.

axr2 rib1 double mutants

A phenotype appeared in the *axr2 rib1* double heterozygote mutants (F1 generation following a cross) that was not seen in *rib1*, wild-type or *axr2* single mutants: the double mutant cotyledons show hyponastic growth, that is the lower face (abaxial) of the cotyledons seems to have grown faster than the upper (adaxial) face, resulting in upcurled cotyledons. This phenotype was not noticed in other mutant or double mutant combinations, and constitutes a novel phenotype. Mutants that overproduce auxins, transgenics overproducing auxin (such as 19S::IaaM plants, that express a bacterial gene resulting in 4-fold higher levels of IAA), and seedlings treated with exogenous auxin usually present the converse phenotype: their cotyledons are epinastic (or down-turned) (Boerjan, et al., 1995; Celenza, et al., 1995; King, et al., 1995; Romano, et al., 1995). This can be interpreted as evidence for differential sensitivity of tissues from the adaxial and abaxial face of the cotyledons, and increased growth of the adaxial face of cotyledons in response to auxins. Interestingly, the *axr2 rib1* double mutants show the opposite phenotype, and could therefore be ascribed to a decreased effect of auxin in the cotyledons. This decrease in auxin signal could be due to reduced auxin levels, modification in the distribution of auxin in this tissue or to reduced auxin signaling. A combination of these effects probably causes the *axr2 rib1* hyponastic cotyledon phenotypes, as the *axr2* mutant is affected in the *IAA7* gene important for auxin signaling (Nagpal et al., 2000), and the *rib1* is known to affect IBA transport and therefore probably distribution in seedlings (see Chapter 4).

eir1 rib1 double mutants

eir1 has a wild-type response to IBA both in root elongation assays and root bending assays (Chapter 2), suggesting the efflux carrier this gene encodes is specific for IAA transport and does not transport IBA. *eir1 rib1* double mutants show that even though *rib1* does not affect ACC response, it can suppress the ACC resistance of *eir1* roots. The double mutant is also much more resistant to 2,4-D than the *rib1* single mutant, which is surprising since *eir1* confers a slightly increased 2,4-D sensitivity

compared to wild-type, and root bending assays suggest 2,4-D (like IBA) is not transported by the *EIR1* encoded carrier. On the other hand, *eir1* and *rib1* had additive effects for IBA and IAA resistance (double mutants were equally IBA resistant as *rib1* single mutants, and *eir1* alone did not affect IBA resistance; single and double mutants had wild-type sensitivity to IAA). Although *rib1* does not directly affect IAA transport (all flows of IAA transport occur normally in *rib1* seedlings), it abolishes the ability of NPA to reduce root acropetal IAA transport, indicating it can affect the regulation of IAA transport. Our results suggest a reduction in IAA basipetal transport (by *eir1* - see Chapter 3) coupled to the *rib1* mutation specifically affect the response to 2,4-D and ACC. Investigation of the transport of IAA, IBA and 2,4-D in the double mutants should help shed some light on these intriguing results.

hy5 rib1 double mutants

In two different families of *hy5 rib1* mutants, carrying different *hy5* mutations in different background ecotypes, F2 individuals that were much more resistant to 2,4-D than either single mutant were observed. These individuals are very significantly longer than either single mutant parent strains: on 2,4-D, the roots of double mutants are 2 or 2.5-fold longer than *hy5-215* or *rib1*, respectively. Approximately 1 16^{th} (one sixteenth) of F2 individuals showed very high levels of 2,4-D resistance, suggesting synergism requires homozygosity at both *hy5* and *rib1* loci despite the fact that *rib1* is semi-dominant for 2,4-D resistance. Though the *hy5* long hypocotyl phenotype is recessive, we have not determined the dominant or recessive nature of auxin resistance conferred by *hy5* mutations. The very high level of resistance of the double mutants suggests *rib1* and *hy5* have synergistic effects.

The putative *hy5 rib1* homozygous lines also displayed novel phenotypes not seen in either single mutant: reduced plant size with loss of apical dominance, reduction in seed set with some seedling mortality, and occurrence of rare seedlings lacking basal structures.

The lack of root and hypocotyl in some *hy5 rib1* double mutants is reminiscent of the phenotype of the *monopteros*, *bodenlos* and *axr6* mutants, which also lack basal

portions of the seedling (Berleth and Jürgens, 1993; Hamann et al., 1999; Hobbie et al., 2000 - see also Table 1.3). Interestingly *MONOPTEROS* encodes an auxin response factor (ARF5) and *BODENLOS* encodes an Aux/IAA protein (IAA12) that could interact in certain cells (Hamann et al., 2002; Hardtke and Berleth, 1998). This suggests MP/ARF5 and BDL/IAA12 proteins act together to mediate auxin signaling necessary for proper specification of the basal structures of the seedling. *axr6* is also affected in auxin signaling: it encodes the cullin subunit of SCF type E3 enzymes (Hellmann et al., 2003). Similar phenotypes are seen in *axr1 rce1* double mutants (2 enzymes involved in RUB modification of cullin - see Figure 1.6), suggesting RUB1 conjugation is required for auxin dependent patterning of the embryo (Dharmasiri et al., 2003). The no root and or no hypocotyl phenotypes of *rib hy5* double mutants suggests these mutations could also affect auxin signaling for basal tissue specification, though more thorough analysis of segregation of these phenotypes would be required to determine the exact inheritance pattern, including the degree of penetrance and the level of expressivity of this phenotype in different lines.

Taken together, the similarities in phenotypes (long hypocotyls, increased lateral root formation and auxin resistance) and our analysis of *hy5 rib1* double mutants suggest *rib1* and *hy5* could be functionally redundant, act in the same pathway or that their function could overlap in many aspects of development: embryo development, hypocotyl response to light, root response to auxin, and apical dominance. Interestingly, all these phenotypes have been associated with defects in auxin signaling in other plants; for example, the *axr1* auxin response mutant presents a short and bushy phenotype (Estelle and Somerville, 1987), and the above-mentioned *mp* and *bdl* mutants, affected in an ARF and an Aux/IAA protein, respectively, lack basal structures of the seedling (Hamann, et al., 2002; Hardtke and Berleth, 1998). The *hy5* locus has mostly been studied for its role in light response, and HY5 is mainly considered as a positive regulator of photomorphogenesis, though it has been suggested to act in auxin response because it affects lateral root formation and elongation, and root gravity response (Oyama, et al., 1997). Our results clearly support a role for the HY5 transcription factor in auxin response, and suggest partial redundancy with *rib1* for many responses. Further analysis of the double mutant, for example by studying gravity response, lateral root formation,

root hair formation and light response in double mutants would reveal whether HY5 and RIB1 interact for all these phenotypes.

rty1 rib1 double mutants

rib1 is able to partially suppress the excessive root production caused by the *rooty1-5* mutation (Figure AI.3). The phenotypes of *rty1-5* and of the allelic *sur1, alf1, hls3,* and *rty1-1* are thought to be caused by increased auxin accumulation due to a defect in regulation of an auxin biosynthetic gene (Boerjan et al., 1995; Celenza et al., 1995; Gopalraj et al., 1996; King et al., 1995, Windsor, 2001). Interestingly, increased efflux of exogenously applied auxin was shown to occur in *sur1* mutants, indicating this mutation also affects auxin transport, probably as a consequence of the morphological or physiological changes induced by increased auxin accumulation (Delarue, et al., 1999). The weaker *rooty* phenotype of individuals produced following a cross to *rib1* indicate *rib1* can partially suppress the *rooty* phenotype. This suppression could be due either to decreased auxin signaling, or to a change in the distribution of auxin in the *rib1* background. As *rib1* was shown not to affect IAA response or transport in seedlings, our results could indicate changes in IBA accumulation or distribution can alleviate the *rooty* phenotype. Though IAA levels have been measured in *rooty* individuals, IBA levels have not. It would be interesting to determine IBA levels in *rty1* to see whether some of the effects attributed to IAA could in fact be due to IBA accumulation, and whether *rib1* affects accumulation or distribution of either auxin in a *rty1* background. On the other hand, crosstalk between IAA and IBA transport, signaling or regulatory pathways could also occur, and explain suppression of the *rty* phenotype by *rib1*.

tir2 rib1 double mutants

Very little has been published about *tir2*, but its phenotypes suggest a defect in auxin transport. *rib1* also clearly affects IBA transport, and response to NPA (chapters 2 and 4). Our results suggest that these mutations could have a redundant function in flowers resulting in defects in anther development/opening when both are mutated. These

results are consistent with a model in which *rib1* acts through its effects on auxin distribution.

Materials and Methods

Plant growth conditions were as described previously (Poupart and Waddell, 2000 - see Chapter 2). *aux1-7, axr1-3, axr2-1, axr4-2, eir1-1* and *hy5-1* seeds were obtained from the Arabidopsis stock center. *hy5-215* seeds were graciously provided by Christian Hardtke (McGill University), and *tir2-1* seed by Mark Estelle (Indiana University, Dept. of Biology). The *rty1-5* allele was isolated in our laboratory and maintained as a heterozygote, and the 16-10C line was used for crosses (line described in Windsor 2001)

Generation and identification of double mutants:

Crosses were made between *rib1* and these mutants using standard techniques, and phenotypes were observed in the F1, F2 and F3 generations. For the recessive mutations *aux1-7, axr1-3,* and *axr4*, double mutants were identified in the following manner: the homozygous recessive mutants were used as maternal strains and *rib1* homozygotes were used as the paternal strain. The F1 were plated on 5 $X10^{-7}$ M IAA, and sensitive individuals were selected based on a short root phenotype, thereby ensuring that a cross had occurred (as *rib1* does not confer IAA resistance). The sensitive individuals were transferred to soil and grown to produce seeds. IAA resistant individuals were isolated in the F2 generation: these were homozygous for the recessive mutation. To identify plants that were also homozygous for *rib1*, we crossed these individuals to wild-type. The progeny of these crosses were plated on 2,4-D. F2 individuals homozygous for *rib1* were found based on the fact they produced only 2,4-D resistant seedlings when crossed to wild-type (as the *rib1* mutation is semi-dominant). A similar approach was used to identify *eir1 rib1* double mutants, but ACC resistance was used instead of IAA to find homozygous *eir1* individuals. As *axr2* confers IAA resistance in a dominant fashion, a different approach was used to identify *axr2 rib1* double mutants. Following a cross between homozygous *axr2* (female parent) and *rib1* mutants (male parent), the F1 individuals were both IAA and 2,4-D resistant. We noted that the cotyledons of the F1 individuals folded on themselves (they were hyponastic).

The progeny of these F1 lines segregated IAA resistant and sensitive individuals in an approximately 3:1 ratio. The fact that IAA resistance segregated in F2 lines insured a cross had occurred. The hyponastic cotyledon phenotype was inherited in the following generations, and segregated, though the segregation pattern was not carefully analysed, as insufficient numbers of plants were generated. Double homozygous *axr2 rib1* mutants were unfortunately not clearly identified and further segregation analysis will be required to recover these. We found NPA resistance of *tir2* to be too weak to use to follow the mutation, and we therefore used *tir2* as the maternal parent, and *rib1* as pollen donor. As *tir2* does not confer 2,4-D resistance, we ensured that a cross had occurred by selecting 2,4-D resistant F1 individuals to generate the F2 generation. Because we could not rely on *tir2* NPA resistance to follow the mutation, we used a PCR approach to identify putative *tir2 rib1* double mutants. We selected F2 individuals that were homozygous for Columbia PCR markers nF5I14 at positions I-92.1 and nga111 at position I-115.5, two markers that flank the position of the *tir2* mutation. As these markers are 23.4 cM apart, the chance of having a double recombination event occurring between them would be approximately 5.5 % (23.4% X 23.4%), so we calculate that more than 94.5% of the individuals recovered should be homozygous Col-0 for the *tir2* locus, and therefore homozygous *tir2* mutants. Homozygosity at the *rib1* locus was determined based on 2,4-D resistant root growth of the progeny of putative *tir2* homozygotes identified as described above. *hy5 rib1* double mutants were identified by the higher level of 2,4-D resistance of double mutants relative to either parental strain. In the *hy5-1* (Ler background) family tested, 8 out of a total of 143 F2 individuals had very long roots on 2,4-D, a value consistent with these individuals representing *hy5 rib1* double homozygotes (expected number, 1 / 16 = 8.94 / 143, Chi-square *P* value = 0.75). Similar results were obtained in *hy5-215* (Col-0 background) F2s: 15 out of 250 F2 individuals had very long roots (expected number 15.625 / 250, Chi-square *P* value = 0.67). *rty1 rib1* double mutants were generated in the following fashion: homozygous *rib1* individuals (female parent) were crossed to heterozygous *rty1* individuals (male parent). Approximately one half of the F1 individuals were heterozygous at both loci, and both 2,4-D resistance and *rty* individuals segregated in the F2 generation. Lines in which *rty* individuals could be seen were carefully examined, and amongst these, individuals with

weak, intermediate and strong *rty* phenotypes were seen, as depicted in Figure AI.2. All classes of *rty* individuals were transferred to soil, including nine with weak *rty* phenotypes. Only one weak *rty* grew to produce flowers and set seed. This plant was dwarf, had strongly epinastic leaves and produced few flowers. From this plant, a single silique contained ten seeds that were sown on GM medium. As all these seeds either did not germinate or presented a *rty* phenotype, we concluded that this plant was homozygous for *rty*, yet partially rescued by the *rib1* mutation so that it could develop past the seedlings stage (homozygous *rty1-5* individuals never produce flowers or seeds in the absence of other mutations). None of the progeny of the partially rescued weak *rty* individual grew beyond the seedling stage, suggesting variable expressivity of the rescue phenotype.

Hypocotyl and root growth measurements:

As a first step to characterize the hormone and inhibitor resistance of double mutants, root elongation was measured on 7 day-old seedlings after plating seeds directly on GM medium containing different hormones and inhibitors. Stocks and dilutions were made according to our standard protocols (see Chapter 2). The following concentrations of hormones or inhibitor were added to plates: 6 X 10^{-8} M 2,4-D, 5 X 10^{-7} M IAA, 3 X 10^{-6} M IBA, 10^{-6} M ACC, 5 X 10^{-7} M NAA, 10^{-5} M ABA, 5 X 10^{-6} M NPA. Plates were placed vertically in white (2,4-D) or yellow filtered light (others) to prevent possible photo-degradation of compounds (Stasinopoulos and Hangarter, 1990). The concentrations of hormones and inhibitors were chosen by analyzing the dose response curves of wild-type roots to these compounds in root transfer assays, such as those presented in Chapter 2 (Poupart and Waddell 2000), and also by empirical determination of the concentrations at which differences between wild-type and mutants were easiest to see with an assay in which seedlings were directly plated on hormone containing plates. Wild-type, single mutants and double mutants were plated on the same plate when possible, or on plates made from the same stocks and media to ensure minimal plate to plate variation. Only Columbia wild-type was used in the assays presented in Figure AI.3, as these were preliminary assays designed primarily to determine whether

differences between single and double mutants could be detected, and all genotypes were placed on the same plate. For the data presented in Table AI.1, we calculated root growth of single and double mutants relative to growth on no hormone in most cases. Root length was determined in the following manner: seedlings were transfered to transparencies, magnified using a retroprojector and traced onto paper. The tracings were then digitalized using a scanner and measured using the NIH image program, as previously described (Poupart and Waddell, 2000, Chapter 2 of this thesis).

The length of hypocotyls of 7 day-old wild-type, single and double mutant seedlings was measured using the same techniques as root length measurements described above. White and red light conditions were as described in Chapter 4. Media used was standard GM, containing 1% sucrose and 0.8% Difco agar.

Statistical analysis:

Statistical analysis was done using Microsoft Excel. Student's t-tests assuming equal or unequal variance were used as appropriate to determine the significance of differences between wild-type, single and double mutants.