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GENETIC ANALYSIS OF AUXIN HOMEOSTASIS: CONJUGATE SENSITIVITY AND AUXIN SUPERSENSITIVITY

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

DOCTOR OF PHILOSOPHY

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ABSTRACT

Genetic analysis of auxin homeostasis:
conjugate sensitivity and auxin supersensitivity

by

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Through their regulation of gene expression and cell growth, auxins affect many aspects of plant growth and development. One mechanism through which auxin homeostasis is achieved is by conjugating indole-3-acetic acid (IAA, the most common natural form of auxin) to amino acids or sugars. The Arabidopsis mutant ilr2-1 was isolated as an IAA-Leucine resistant mutant that retains wild-type sensitivity to free IAA. ilr2-1 is resistant to IAA-Leu and IAA-Phe, but not to other conjugated forms of IAA. It is also resistant to cobalt and manganese. ilr2-1 has fewer lateral roots than wild type, which can be rescued by exogenous IAA conjugates; and it has a shorter root than wild type, which can be rescued by exogenous metals, including cobalt. Using a map-based approach, I cloned the ILR2 gene, which encodes a novel protein that is polymorphic between Arabidopsis ecotypes. A T-DNA insertion in the second form of ILR2, ilr2-2, results in cobalt supersensitivity. The ILR2 transcript appears to be expressed at very low levels, and accumulates in aerial tissues and in response to exogenous cobalt.

Auxin resistant mutant screens have provided valuable information about how auxin is sensed and regulated; they also suggest that a considerable part of auxin homeostasis is dependent on negative regulation. To identify these negative regulators, we developed a genetic screen that allows the identification of three auxin supersensitive
(axs) mutants. These mutants exhibit abnormal responses in conditions leading to auxin accumulation, such as auxin transport inhibitors and growth at high temperatures. We used recombination mapping to identify the genes defective in these mutants. The axs1 mutant that is resistant to low concentrations of auxin transport inhibitors, and the axs2 mutant that has a higher number of lateral roots than wild type. Both mutations were mapped, based on a defective root-curling phenotype, to the region in chromosome I contained between the markers nga280 and nga111: this mapping location may represent a QTL involved in root-curling. The axs3 mutant, supersensitive to high temperature, was mapped to the region in chromosome I contained between the markers nga62 and nga280. The analysis of mutants such as axs3 is expected to contribute to our understanding of how auxin levels are spatially and temporally regulated in the plant.
ACKNOWLEDGMENTS

I want to thank my advisor, Dr. Bonnie Bartel, who took me in as a “slightly used” graduate student, and gave me two challenging projects to learn from. In addition, I am also thankful for that door that is always open for help.

I want to also thank the members of my thesis committee: Dr. Janet Braam, Dr. George N. Bennett, Dr. Charles R. Stewart and Dr. Ronald J. Parry for their suggestions through the development of this thesis.

In addition, I want to thank the members of the Bartel lab. Andrew Woodward worked with me in the axs project, and gave me the opportunity to teach him while learning from him. Also, current and past members of the lab, specially Raquel Adham, Jamie Lasswell, Sherry LeClere, Melanie Monroe-Augustus, Rebekah Rampie, Luise Rogg, and Bethany Zolman. They have been good friends and a source of support.

I also want to thank the members of my family who taught me how beautiful learning always is. Particularly my parents, Mario Magidin and Elisa Viso, I appreciate all their encouragement and help through the years I have been at Rice, as well as through the many years before.

Finally, I want to thank my husband, Eyal de Lara; without him I probably would have run back to Mexico after the first couple of years in the U.S. and never would have finished my Ph.D. His understanding and supportive ways have been invaluable to me.
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ABBREVIATIONS

2,4-D  2,4-dichlorophenoxyacetic acid

ABA  abscisic acid

ABRC  Arabidopsis Biological Research Center

ACC  1-aminocyclopropane-1-carboxylic acid

AGI  Arabidopsis Genome Initiative

AIB  α-aminoisobutyric acid

ARID  A/T rich interacting domain

ATI  auxin transport inhibitor

AVG  aminoethoxyvinylglucine

BAC  bacterial artificial chromosome

BFA  brefeldin-A

bp  base pairs

CaMV  Cauliflower Mosaic Virus

CAPS  cleaved amplified polymorphic sequences

CDF  cation diffusion facilitator

Col-0  Columbia ecotype

EMS  ethyl methanesulfonate

IAA  indole-3-acetic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tr>
<td>IAA-Ala</td>
<td>IAA-L-alanine</td>
</tr>
<tr>
<td>IAA-Asp</td>
<td>IAA-L-aspartic acid</td>
</tr>
<tr>
<td>IAA-Glu</td>
<td>IAA-L-glutamic acid</td>
</tr>
<tr>
<td>IAA-Glucose</td>
<td>1-β-indole acetyl glucose</td>
</tr>
<tr>
<td>IAAld</td>
<td>indole-3-acetaldehyde</td>
</tr>
<tr>
<td>IAA-Leu</td>
<td>IAA-L-leucine</td>
</tr>
<tr>
<td>IAA-Phe</td>
<td>IAA-L-phenylalanine</td>
</tr>
<tr>
<td>IAN</td>
<td>indole-3-acetonitrile</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase pairs</td>
</tr>
<tr>
<td>Ler</td>
<td>Landsberg erecta tt4 ecotype</td>
</tr>
<tr>
<td>Mb</td>
<td>megabase pairs</td>
</tr>
<tr>
<td>MT</td>
<td>methallotineins</td>
</tr>
<tr>
<td>NPA</td>
<td>1-N-naphthylphthamic acid</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>OxIAA</td>
<td>oxindole-3-acetic acid</td>
</tr>
<tr>
<td>PC</td>
<td>phytochelatins</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PN</td>
<td>plant nutrient media</td>
</tr>
<tr>
<td>PNS</td>
<td>plant nutrient media with sucrose</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>QTL</td>
<td>quantitative trait loci</td>
</tr>
<tr>
<td>RACE PCR</td>
<td>rapid amplification of cDNA ends PCR</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase PCR</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenyl methionine</td>
</tr>
<tr>
<td>SSLP</td>
<td>simple sequence length polymorphism</td>
</tr>
<tr>
<td>T-DNA</td>
<td>transferred DNA</td>
</tr>
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<td>TIBA</td>
<td>2,3,5-triiodo-benzoic acid</td>
</tr>
<tr>
<td>Ws</td>
<td>Wassilewskija ecotype</td>
</tr>
<tr>
<td>YAC</td>
<td>yeast artificial chromosome</td>
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CHAPTER I. BACKGROUND

This thesis analyzes proteins important for control of the phytohormone auxin, through the identification and characterization of mutants with impaired functions in different aspects of auxin homeostasis. Auxins were the first class of hormones identified in plants because of their regulatory role in cell growth resulting from phototropism (Skoog 1937). Indole-3-acetic acid, or IAA, is the most common naturally occurring auxin. IAA has been implicated in a variety of plant processes ranging from embryo development (Bennett et al. 1995), lateral root formation (Celenza et al. 1995), and gravitropism (Maher and Martindale 1980) to apical dominance and leaf shape (Chatfield et al. 2000). At the cellular and molecular level, auxins control cell turgor, elongation, division, and they influence gene expression (Estelle 1999). Despite the numerous effects of auxin, the complete molecular mechanisms of auxin homeostasis and action remain unidentified. The fact that plants regulate the concentration of free IAA by de novo synthesis, conjugation to amino acids or sugars, deconjugation, transport and catalysis (Figure I-1) adds to this complexity (Bartel et al. 2001). Using genetic and reverse genetic tools, the proteins involved in some of the important aspects of auxin regulation and response have been identified.

This thesis characterizes Arabidopsis mutants involved in the regulation of IAA homeostasis. Therefore, the following sections discuss the use of Arabidopsis as a model system, the mechanisms mediating the auxin response, and the different pathways involved in the maintenance of IAA homeostasis: IAA biosynthesis, IAA conjugation and
deconjugation, IAA transport, and IAA catabolism. The study of IAA-conjugate mutants led to the identification of a link between metals and IAA (this work, Lasswell et al. 2000); therefore, the last section of this introduction discusses the regulation of metal homeostasis and its relation with hormone action.

![Diagram](image)

**Figure I-1. Regulation of auxin homeostasis.** The levels of active auxin, or free IAA, are regulated temporally and spatially by the indicated pathways.
I.A. Arabidopsis as a model system

For the genetic studies discussed in this thesis, we chose to use the model plant Arabidopsis thaliana. Arabidopsis is used worldwide as a model organism to study plant processes because its small size, rapid growth, low chromosome number, and high fertility make it amenable to genetic studies (Meyerowitz 2001). The ease of work with Arabidopsis has led to the development of communal tools, including polymorphic markers based on the polymerase chain reaction (PCR) used for genetic mapping (Konieczny and Ausubel 1993; Bell and Ecker 1994), a comprehensive list of polymorphisms between the Col-0 and Ler ecotypes (http://www.tair.org/cereon), a simple transformation technique (Clough and Bent 1998), and genomic libraries constructed in yeast and bacterial artificial chromosomes (YAC and BAC; Rede and Koncz 1992). Furthermore, the Arabidopsis Genome Initiative (AGI) has sequenced the 120-Mb Arabidopsis genome, which makes the use of Arabidopsis in a genetic screen even more desirable. In addition, several collections of mutagenized Arabidopsis lines are available to the community, allowing the isolation of mutants in specific desired genes, whether resulting from T-DNA insertions (Young et al. 2001) or a novel method that identifies base-pair mutations (Colbert et al. 2001). The use of Arabidopsis as a genetic model system has greatly aided the understanding of a variety of physiological responses at the molecular level and, of special concern for this thesis, how plants regulate and respond to auxin.
I.B. IAA conjugation and deconjugation

One important mechanism for the maintenance of IAA homeostasis is the conjugation and deconjugation of free IAA through its carboxyl group to small molecules such as amino acids or carbohydrates (Figure 1-2; Bandurski et al. 1995; Tam et al. 2000). In fact, 90% of the IAA found in Arabidopsis is in an amide-conjugated form, approximately 10% is in ester-linked IAA conjugates, and only a small part of IAA is in the free, presumably active, form (Tam et al. 2000). Conjugated forms of IAA are generally thought of as inactive forms of the hormone used for storage, transport, or as catabolic intermediates (Normanly and Bartel 1999). Part of this thesis is the study of the IAA-conjugate-resistant mutant ilr2; therefore, this section covers the importance that the IAA conjugation and deconjugation branch has in IAA homeostasis. In particular, this section discusses the types of conjugates that have been identified from plants, their proposed role, and the known genes and proteins important for this branch of IAA homeostasis.
Figure 1-2. The majority of IAA found in plants is conjugated to esters or amino acids. In Arabidopsis IAA esters represent approximately 10% of total IAA and IAA-amino acids represent 85 to 90% of total IAA (Tam et al. 2000).

I.B.1. Conjugates identified from plants

All analyzed plants contain different forms of IAA conjugates, but the ratio of amide to ester conjugated IAA and the specific types of IAA conjugates varies between plant species. In general, monocots contain a higher proportion of ester conjugates, and dicots accumulate mostly amide conjugates (Cohen and Bandurski 1982; Slovin et al. 1999). Conjugates that have been identified from plants include IAA-Asp and IAA-Glu from soybeans (Epstein et al. 1986); IAA-\textit{myo}-inositol, IAA-\textit{myo}-inositol glycosides, and 1-\textit{o}-indole acetyl glucose (IAA-glucose) from maize (Cohen and Bandurski 1982); and

In addition to conjugates formed with esters or amino acids, a form of IAA conjugated with a peptide has been identified from Arabidopsis (Walz et al. 2001). Other forms of IAA conjugated with several polypeptides of varying sizes have been identified in bean seeds (Bialek and Cohen 1986; Walz et al. 2001). The polypeptide conjugated to IAA in one of these bean IAA-peptide conjugates was identified as IAP1, a homologue of a soybean late seed maturation protein (Bialek and Cohen 1986; Walz et al. 2001).

I.B.2. Roles of IAA conjugates

Given the variety of IAA conjugates, the moiety to which the IAA is conjugated could dictate its fate in the same way it determines its efficiency in biological assays (Bartel 1997; Bartel et al. 2001). Not all IAA conjugates evoke a response in bioassays, and the general correlation is that conjugates that can be hydrolyzed in planta have assayable auxin effects (Bartel et al. 2001). For example, IAA-Leu and IAA-Ala inhibit primary root elongation in Arabidopsis; ILR1 and IAR3 are two amidohydrolases identified from Arabidopsis that can cleave these conjugates, respectively (Bartel and Fink 1995; Davies et al. 1999). IAA-Ala and IAA-Leu are probably inactive forms of the hormone used for storage that have auxin activity based on their hydrolysis to produce free IAA. IAA-Ala and IAA-Leu may have different and specific roles in the plants as they have been found to accumulate in varying Arabidopsis tissues: IAA-Leu is present at higher concentrations in root tissues, whereas IAA-Ala is present at higher concentrations in aerial tissues (Kowalczyk and Sandberg 2001).
By transporting IAA in the form of conjugates, the plant may prevent the elicitation of auxin-induced responses that would be observed with a more active form of the hormone. IAA-ester conjugates are transported much faster than IAA, possibly through the vasculature (Nowacki and Bandurski 1980; Ludwig-Müller et al. 1996). However, IAA-amino acid conjugates that function as IAA storage forms, namely IAA-Leu and IAA-Ala, apparently enter cells in the same way as IAA does, since an aux1 mutant, defective in the auxin influx carrier (Bennett et al. 1996), is resistant to these conjugates (S. LeClere, B. Bartel, unpublished).

Conjugates may also function in excess IAA detoxification. In fact, IAA is conjugated in response to high exogenous concentrations. Arabidopsis seedlings, in addition to oxidizing exogenous IAA, conjugate it to Asp and Glu; IAA-Asp can then be oxidized as well (Östín et al. 1998). Mutants with defective IAA homeostasis also exhibit abnormal conjugate accumulation. For example, the auxin overproducer sur2 mutant (see section I.E) accumulates lower concentrations of the hydrolyzable conjugate IAA-Leu. In contrast, the sur2 mutant accumulates free IAA as well as the oxidizable conjugates IAA-Asp and IAA-Glu (Barlier et al. 2000), which are not hydrolyzed in Arabidopsis (Östín et al. 1998). Furthermore, rapidly dividing tissues in Arabidopsis, which contain the highest levels of free IAA throughout the plant, also contain higher levels of IAA-Asp and IAA-Glu (Kowalczyk and Sandberg 2001). These results further support the hypothesis that the different forms of conjugates identified in plants serve different in vivo functions (Bartel et al. 2001).
I.B.3. Genetic analysis of IAA conjugate homeostasis

One mechanism used to better understand the role of auxin conjugates is by studying mutants that have abnormal responses to these conjugates. Auxins inhibit primary root elongation when present at high concentrations (Maher and Martindale 1980). Certain IAA conjugates, including IAA-Leu, IAA-Ala, and IAA-Phe, have the same effect on Arabidopsis seedlings (Bartel and Fink 1995; Davies et al. 1999). This effect has been used as the basis for genetic-based approaches, which have provided an important tool to understand IAA conjugate biology. The known genes and mutants implicated in IAA conjugate homeostasis are listed in Table I-1.

Table I-1. Plant genes implicated in IAA conjugate metabolism. (modified from Bartel et al. 2001).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Product</th>
<th>Putative Localization</th>
<th>Mutant Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ILR1</td>
<td>Arabidopsis</td>
<td>IAA-amino acid amidohydrolase</td>
<td>ER lumen</td>
<td>IAA-Leu resistant</td>
<td>(Bartel and Fink 1995)</td>
</tr>
<tr>
<td>IAR3</td>
<td>Arabidopsis</td>
<td>IAA-amino acid amidohydrolase</td>
<td>ER lumen</td>
<td>IAA-Ala resistant</td>
<td>(Davies et al. 1999)</td>
</tr>
<tr>
<td>ILL1</td>
<td>Arabidopsis</td>
<td>IAA-amino acid amidohydrolase</td>
<td>ER lumen</td>
<td>not reported</td>
<td>(Bartel and Fink 1995)</td>
</tr>
<tr>
<td>ILL2</td>
<td>Arabidopsis</td>
<td>IAA-amino acid amidohydrolase</td>
<td>ER lumen</td>
<td>not reported</td>
<td>(Bartel and Fink 1995)</td>
</tr>
<tr>
<td>IAR1</td>
<td>Arabidopsis</td>
<td>transporter?</td>
<td>membrane</td>
<td>IAA-amino acid conjugate</td>
<td>(Lasswell et al. 2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>resistant</td>
<td></td>
</tr>
<tr>
<td>ILR2</td>
<td>Arabidopsis</td>
<td>Novel gene</td>
<td>unknown</td>
<td>IAA-Leu resistant</td>
<td>This thesis</td>
</tr>
<tr>
<td>IAR4</td>
<td>Arabidopsis</td>
<td>Pyruvate dehydrogenase</td>
<td>not reported</td>
<td>IAA-Ala resistant</td>
<td>(LeClere and Bartel, unpublished)</td>
</tr>
<tr>
<td>ICR1</td>
<td>Arabidopsis</td>
<td>not reported</td>
<td>not reported</td>
<td>IAA-Phe resistant</td>
<td>(Campanella et al. 1996)</td>
</tr>
<tr>
<td>ICR2</td>
<td>Arabidopsis</td>
<td>not reported</td>
<td>not reported</td>
<td>IAA-Phe resistant</td>
<td>(Campanella et al. 1996)</td>
</tr>
<tr>
<td>iaglu</td>
<td>Zea mays</td>
<td>IAA glucosyltransferase</td>
<td>not reported</td>
<td>not reported</td>
<td>(Szerszen et al. 1994)</td>
</tr>
<tr>
<td>UGT84B1</td>
<td>Arabidopsis</td>
<td>IAA glucosyltransferase</td>
<td>not reported</td>
<td>not reported</td>
<td>(Jackson et al. 2001)</td>
</tr>
<tr>
<td>IAP1</td>
<td>Phaseolus vulgaris</td>
<td>seed protein modified by IAA</td>
<td>not reported</td>
<td>not reported</td>
<td>(Walz et al. 2001)</td>
</tr>
</tbody>
</table>
A family of IAA conjugate amidohydrolases was identified from Arabidopsis by identifying mutants that are resistant to IAA conjugates but retain wild-type sensitivity to free IAA (Bartel and Fink 1995; Davies et al. 1999). ILR1 hydrolyzes IAA-Leu and ilrl mutants are IAA-Leu resistant (Bartel and Fink 1995). Similarly, IAR3 hydrolyzes IAA-Ala and iar3 mutants are IAA-Ala resistant (Davies et al. 1999). Based on the similarity to the amidohydrolase ILR1, other genes from this family have been identified. These include ILL1, ILL2, ILL4, ILL5 and ILL6/GR1 (for ILR-1 like). The ILL, IAR3, and ILR1 proteins have been heterologously expressed and analyzed in vitro to determine their ability to hydrolyze IAA conjugates (LeClere et al. 2001). Most amidohydrolases exhibit higher activity towards IAA-Ala than IAA-Phe or IAA-Leu, except for ILR1, which cleaves IAA-Phe and IAA-Leu while exhibiting little or no activity towards IAA-Ala (LeClere et al. 2001). Their K_m towards their preferred conjugate varies greatly with ILL2 exhibiting a K_m of 52 µM for IAA-Ala, ILR1 with a K_m of 8 µM for IAA-Leu, and ILL1 exhibiting little activity towards any tested IAA conjugates (LeClere et al. 2001).

The ILL6/GR1 gene was identified because its mutation results in higher susceptibility to the powdery mildew Peronospora (GenBank accession AJ010735), and the IAR3 gene was also isolated as JR3, a gene induced in response to wounding (Leon et al. 1998; Rojo et al. 1998). These results, together with the variation in their enzymatic activity for IAA conjugates, suggest that the biological activity of some of these hydrolases may not be related to IAA conjugates directly. Alternatively, they may indicate a relation between auxin conjugates and pathogen responses.

In addition to ilrl and iar3, iar1 is a third IAA-amino acid conjugate resistant mutant. IAR1 encodes a protein with seven predicted transmembrane domains that shares
regions of similarity with the ZIP family of metal transporters, thus suggesting a role for ions in regulating IAA-conjugate cleavage or activity (Lasswell et al. 2000). The iar1 mutant’s IAA-conjugate resistance can be rescued by manganese; however, no altered ion sensitivity or ion content was detected in the mutant (Lasswell 2000).

A fourth mutant, iar4, was isolated as an IAA-Ala resistant mutant. This short-rooted mutant is also resistant to auxin, auxin transport inhibitors, cytokinin, and a variety of other hormones (S. LeClere, personal communication). The gene defective in this mutant was cloned and identified as a putative pyruvate dehydrogenase (S. LeClere, personal communication).

Lastly, the icr1 and icr2 mutants are also resistant to several IAA-amino acid conjugates (Campanella et al. 1996), but the genes defective in these mutants have not been reported.

The analysis of mutants defective in IAA conjugate metabolism has been instrumental to the present understanding of this mechanism. The enzymes responsible for IAA-amino acid conjugate hydrolysis were identified through this method (Bartel and Fink 1995; Davies et al. 1999; LeClere et al. 2001). Furthermore, the mechanisms that may regulate the hydrolysis of IAA-amino acid conjugates are starting to be identified by the non-biased genetic approach that has shown the possible link between IAA and metal homeostasis. This link is further explored in this thesis through the study of ilr2, an IAA-Leu resistant mutant.
I.C. IAA transport

Auxin transport constitutes an important mechanism for regulating auxin homeostasis. The amount of active auxin present in a given tissue can be regulated by adjusting the quantity of auxin coming in or out of this tissue. Genes defective in auxin-transport mutants have been identified and their characterization has been essential in providing long awaited proof to classic models of auxin transport (Estelle 1998; Jones 1998). These genes are listed in Table I-2.

AUX1 is an auxin influx carrier, identified through the study of the auxin-resistant mutant $aux1$, which also exhibits agravitropic roots (Picket et al. 1990; Bennett et al. 1996). Since mutations that inhibit auxin influx into the cell result in auxin resistance, the need for a classical membrane localized auxin receptor is obviated. It is not known whether auxin influx is regulated, but given that IAA can also enter the cell by diffusion when protonated, it seems unlikely.

In contrast to auxin influx, auxin efflux appears highly regulated. A family of genes coding for auxin efflux carriers has been identified by homology with the genes defective in the mutants $pin1$ and $eir1$. The $pin1$ mutants, defective in a shoot auxin efflux carrier, exhibit decreased auxin transport, probably resulting in auxin accumulation in the shoot inflorescences that terminate in pin-like structures and lack lateral organs. The $PIN1$ gene encodes for a protein similar to membrane carrier proteins, homologous to $EIR1$ (Galweiler et al. 1998). Mutations in $EIR1$ were initially isolated by their ethylene insensitive root, which was later identified as a result of root-specific defective auxin transport (Roman et al. 1995; Luschnig et al. 1998). The $PIN/EIR1$ family contains
other members localized to specific cellular membranes thus regulating basipetal, acropetal, and lateral auxin transport (Müller et al. 1998).

The PINOID (PID) serine-threonine protein kinase has also been implicated in the regulation of auxin transport. As its name implies, the pinoid phenotype is very similar to the pin-formed phenotype observed in pin mutants. Overexpression of the auxin-inducible PID protein results in root and shoot auxin-resistant phenotypes (Christensen et al. 2000) and enhanced auxin transport (Benjamins et al. 2001), implying that PID may act as a negative regulator of auxin signaling or a positive regulator of auxin transport.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Gene product</th>
<th>Known hormone effects</th>
<th>Screen used for identification</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>auxl</td>
<td>Homology to membrane amino acid permease</td>
<td>Auxin, ethylene, ATP* and partial cytokinin resistance</td>
<td>Auxin-resistant roots</td>
<td>(Pickett et al., 1990; Timpte et al., 1995)</td>
</tr>
<tr>
<td>eirl, agrl, wav6, AtPIN2</td>
<td>Transmembrane protein with similarity to bacterial efflux carriers; root specific</td>
<td>Ethylene and ATP resistance</td>
<td>Ethylene insensitivity and agravitropic root.</td>
<td>(Chen et al. 1998; Luschnig et al. 1998; Müller et al. 1998)</td>
</tr>
<tr>
<td>pinl</td>
<td>EIR1 homolog</td>
<td>Extreme apical dominance</td>
<td>Shoot phenotype</td>
<td>(Gälweiler et al. 1998)</td>
</tr>
<tr>
<td>rcnl</td>
<td>Regulatory A subunit of serine/threonine phosphoprotein phosphatase 2A</td>
<td>Only auxin resistance</td>
<td>Abnormal effect of NPA (an ATI)</td>
<td>(Garbers et al., 1996)</td>
</tr>
</tbody>
</table>


I.C.1. Auxin transport inhibitors

The regulated portion of auxin transport, auxin efflux, appears amenable to biochemical inhibition by a group of compounds known collectively as "auxin transport inhibitors" (ATIs). The classical ATIs include naphthylphtalamic acid (NPA) and 2,3,5-triodobenzoic acid (TIBA). In addition, it has been suggested that naturally-occurring
amino acyl-β-naphthylamide conjugates can also act as auxin transport inhibitors (Murphy et al. 2000). NPA is thought to act by binding to a component of the auxin efflux machinery, not to the transporter itself. A possible target was thought to be the TIR3/DOC1/BIG1 protein, a callosin-like protein involved in directional secretion (Gil et al. 2001). This hypothesis was based on the fact that big1 mutants exhibit reduced NPA binding (Ruegger et al. 1997). However, in big1 mutants the subcellular distribution of the auxin efflux carrier PIN1 is altered, which may be the reason for the reduced NPA binding (Gil et al. 2001).

The cellular localization of PIN1 is very dynamic; it changes constantly between plasma membrane and endosomal compartments (Geldner et al. 2001). The proper cycling of PIN1 is dependent on the membrane-associated GNOM guanine-nucleotide exchange factor (Steinmann et al. 1999). GNOM regulates vesicle transport and is sensitive to the drug brefeldin-A (BFA), which when applied to Arabidopsis plants results in an accumulation of PIN1 in the endosome. TIBA treatment followed by BFA treatment results, in contrast, in the accumulation of PIN1 at the plasma membrane (Geldner et al. 2001). Furthermore, application of BFA to plants results in phenotypes similar to ATI-treated plants (Geldner et al. 2001). In conclusion, auxin transport inhibitors appear to act by blocking directional vesicle transport, which is necessary for the proper localization of auxin efflux carriers.

Despite this direct effect of ATIs on directional vesicle transport, their indirect effect on auxin transport has been used to identify components of the auxin transport machinery. When Arabidopsis seedlings are grown in petri plates, their roots grow towards the bottom of the plate, and then curl in a characteristic pattern. The ATIs inhibit
this root-curling pattern, and auxin-resistant mutants do not exhibit it. The \textit{rcnl} mutant (roots curl on \textit{NPA}), defective in a protein phosphatase A subunit, has the opposite phenotype: its roots curl in the presence of auxin transport inhibitors (Garbers et al. 1996; Deruere et al. 1999). The \textit{rcnl} mutant also exhibits a delayed gravity response and increased basipetal auxin transport. These observations, in addition to the reduced \textit{NPA} sensitivity, suggest that \textit{RCNl} is a negative regulator of auxin transport or a regulator of auxin transport inhibition (Rashotte et al. 2001). The upstream and downstream components of this signaling network remain to be identified, but will undoubtedly be important for understanding auxin signaling and transport regulation. The use of novel mutant screens, such as the one performed in this thesis, might help identify some of these components.

\textbf{I.D. IAA catabolism}

One additional mechanism that plants use to regulate the amount of active auxin is IAA catabolism. In addition to conjugation with amino acids or sugars to serve as storage forms, excess IAA can be catabolized through three mechanisms: IAA conjugate oxidation, IAA oxidation, and IAA decarboxylation. The oxidation of IAA conjugates was discussed previously in this introduction; this section addresses the oxidation and decarboxylation pathways for IAA.

\textbf{I.D.1. IAA oxidation}

Oxidation of IAA to produce oxindole-3-acetic acid (\textit{OxIAA}) is probably the most significant IAA degradation pathway, because formation of \textit{OxIAA} can account for the majority of the IAA that disappears from the IAA pool (Slovin et al. 1999). \textit{OxIAA} has
been identified from maize, *Brassica rapa*, Arabidopsis, and rice (Östlin et al. 1998; Slovin et al. 1999). An IAA-oxidase activity has been identified in maize root extracts (Beffa et al. 1990), and its activity is induced by light or the presence of its cofactors manganese and *p*-coumaric acid (Beffa et al. 1990; Liu et al. 1996). The IAA oxidase is probably a dioxygenase that catalyzes the oxidation of IAA and a fatty acid since oleic, linoleic, and linolenic acids can serve as cosubstrates (Slovin et al. 1999). The identity of the IAA oxidase remains obscure, but given the importance of this pathway, it would be of great interest to identify the involved enzymes. The screen for auxin-supersensitive mutants described in this thesis was aimed at the identification of proteins involved in pathways that, like IAA oxidation, are important for the negative regulation of IAA.

### I.D.2. IAA decarboxylation

An additional pathway for IAA catabolism involves the decarboxylation of IAA. This step is thought to be catalyzed by oxidases, which catalyze the following reaction *in vitro*: indole-3-acetic acid $\rightarrow$ indole-3-methanol + O$_2$ $\rightarrow$ indole-3-aldehyde $\rightarrow$ indole-3-carboxylic acid (Slovin et al. 1999). A plant oxidase activity that can catalyze this reaction has been identified from maize and soybean (Beffa et al. 1990; Liu et al. 1996), but this activity may result from nonspecific decarboxylations. Nevertheless, the products of IAA decarboxylation have been identified *in vivo*, indicating that this process occurs (Slovin et al. 1999). Given that non-decarboxylative oxidation probably represents the major pathway for IAA catabolism, it is tempting to speculate that the decarboxylative pathway plays a specific fine-tuning role in IAA homeostasis. For example, ethylene induces the formation of indole-3-carboxylic acid in *Citrus siniensis* leaves, which may be an important regulation point for ethylene-mediated effects on IAA.
(Saage et al. 1990). Through the detailed examination of auxin-supersensitive mutants, such as those presented in this thesis that are supersensitive to only certain aspects of IAA homeostasis, it may be possible to identify the enzymes involved in this pathway.

I.D.3. IAA oxidative decarboxylation

Through the detailed analysis of the root apical meristem, a third pathway for IAA catabolism was recently described (Kerk et al. 2000). This pathway involves the enzyme ascorbate oxidase (AOX), which is a copper-containing “blue oxidase” whose role in plants is ambiguous; its activity is correlated with cell expansion and its expression is induced by auxin (Kisu et al. 1997). In vitro, AOX can decarboxylate IAA, dependent on the presence of the proposed IAA oxidase cofactors manganese and coumaric acid, to produce oxindole-3-methanol (Kerk et al. 2000). Furthermore, preincubation of IAA with AOX leads to abrogation of the effects of IAA in secondary root induction and inhibition of primary root elongation in the radish root bioassay. This activity is probably restricted to the root apex, where the root apical meristem is located and high levels of AOX have been identified (Kerk and Feldman 1995). The restricted location of this activity was further verified by analyzing the ratio of $\text{L}^{-14}\text{C-IAA}$ to $5^{-3}\text{H-IAA}$ after incubation with maize root sections. Through this experiment, Kerk and coworkers found that about 30% of the fed IAA was decarboxylated, and that the IAA decarboxylation activity was localized to the tip section (Kerk et al. 2000).

IAA decarboxylative oxidation at the root tip may affect overall plant morphology and, in particular, lateral root promotion. The formation of lateral roots is closely associated with auxin, but the mechanism by which auxin mediates this phenomenon is
not clearly understood. One model separates lateral root initiation from lateral root growth. It suggests that initiation is dependent on basipetal auxin transport that occurs from the root towards the shoot/root junction, whereas lateral root growth is dependent on acropetal auxin transport from the shoot towards the roots (Casimiro et al. 2001). Supporting this model is the observation that NPA treatment, which results in accumulation of auxin in the apex of the root, inhibits lateral root formation. However, a second model suggests that acropetal auxin transport across the root/shoot junction promotes both initiation and emergence of lateral roots (Reed et al. 1998). This model is based on the observation that treatment of different regions of the root with NPA has different effects on the formation of lateral roots: NPA treatment at the shoot/root junction inhibits the formation of lateral roots, whereas NPA treatment in the root tip does not affect lateral root growth in distant sites (Reed et al. 1998).

Given that high concentrations of IAA inhibit primary and lateral root growth, the accumulation of IAA at the root tip must have an effect on the growth of lateral roots. Trimming the root tips results in enhanced formation of lateral roots in Arabidopsis and radish roots, as does treatment with copper, which enhances AOX accumulation (Esaka et al. 1992). This observation suggests that the high concentration of IAA in the root apical meristem and its vicinity results in an inhibition of lateral root growth (Kerk et al. 2000), which neither of the mentioned two models accounts for.

**I.E. Auxin biosynthesis and IAA homeostasis control**

Some of the material presented in this section has been published (Bartel et al. 2001)
Another important mechanism in the regulation of IAA levels, or any compound, is the rate of biosynthesis. The current state of knowledge of IAA biosynthetic pathways, illustrated in Figure I-3, assumes the existence of two main pathways with multiple branches. One pathway leads to the formation of IAA from Trp, and the second pathway from a Trp-precursor, probably indole-3-glycerol phosphate (Normanly et al. 1995; Bartel 1997; Normanly and Bartel 1999). The existence of multiple routes to the same product may allow precise regulation of IAA biosynthesis, but understanding of this control is limited.

Although several IAA biosynthetic pathways are now established in plant-associated microbes (Costacurta and Vanderleyden 1995; Patten and Glick 1996), no plant IAA biosynthetic pathway has been fully elucidated to the level where all the proteins and compounds involved are identified. Some of the proteins involved in the Trp-dependent pathway have been identified using a genetic approach; these are listed in Table I-3.

This section discusses IAA biosynthetic pathways and their regulation as a way for controlling IAA homeostasis. The use of Trp-dependent versus Trp-independent IAA biosynthesis may modulate the level of IAA produced. A branch of the Trp-dependent pathway, leading to the production of indole-glucosinolates, appears to regulate the flux from Trp to IAA (Barlier et al. 2000; Bak et al. 2001). In addition, a number of auxin-hypersensitive mutants, like those identified in this thesis, have uncovered points of negative regulation for auxin biosynthesis.
Figure I-3. Proposed pathways of de novo IAA synthesis in plants. *De novo* IAA biosynthetic pathways initiate from Trp or Trp precursors. Trp biosynthesis and the P450-catalyzed conversion of Trp to IAOx are chloroplastic, whereas many Trp-dependent IAA biosynthetic enzymes are cytoplasmic. Several points of negative regulation are shown by blunt arrows. The boxed IAN pathway may be limited to families in the Capparales order that make indole glucosinolates (modified from Bartel et al. 2001)
### Table 1-3. Plant genes implicated in de novo IAA biosynthesis. (modified from Bartel et al. 2001).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Product</th>
<th>Putative Localization</th>
<th>Loss-of-Function (LOF) or Overexpression (OE) Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAO1</td>
<td>Arabidopsis</td>
<td>IAAld oxidase</td>
<td>cytoplasm</td>
<td>OE in ry LOF not reported</td>
<td>(Sekimoto et al. 1998; Seo et al. 1998)</td>
</tr>
<tr>
<td>CYP79B3</td>
<td>Arabidopsis</td>
<td>cytochrome P450</td>
<td>chloroplast</td>
<td>not reported</td>
<td>(Hull et al. 2000)</td>
</tr>
<tr>
<td>CYP83B1/SUR2</td>
<td>Arabidopsis</td>
<td>cytochrome P450</td>
<td>cytoplasm</td>
<td>LOF: high IAA, IAAld, and IAA-Asp; normal IAN; low indole glucosinolates. High number of lateral roots, reduced hypocotyl growth. OE: high indole glucosinolates, reduced apical dominance</td>
<td>(Delarue et al. 1998; Barlier et al. 2000; Bak et al. 2001)</td>
</tr>
<tr>
<td>NIT1</td>
<td>Arabidopsis</td>
<td>nitrilase</td>
<td>not reported</td>
<td>LOF: IAN resistant, normal IAA</td>
<td>(Normanly et al. 1997)</td>
</tr>
<tr>
<td>NIT2</td>
<td>Arabidopsis</td>
<td>nitrilase</td>
<td>not reported</td>
<td>OE: increased sensitivity to IAN, normal IAA</td>
<td>(Normanly et al. 1997)</td>
</tr>
<tr>
<td>TDC</td>
<td>C. roseus</td>
<td>Trp decarboxylase</td>
<td>cytoplasm</td>
<td>OE (canola): low indole glucosinolates; OE (tobacco): low Trp</td>
<td>(Chavadi et al. 1994; Guillet et al. 2000)</td>
</tr>
<tr>
<td>ORP</td>
<td>Zea mays</td>
<td>Trp synthase β</td>
<td>chloroplast</td>
<td>LOF: high IAA-X, normal free IAA</td>
<td>(Wright et al. 1991)</td>
</tr>
<tr>
<td>TRP3</td>
<td>Arabidopsis</td>
<td>Trp synthase α</td>
<td>chloroplast</td>
<td>LOF: high IAA-X and IAN, normal free IAA, low Trp LOF: high IGP, IAN, and indole glucosinolates; normal IAA</td>
<td>(Normanly et al. 1993; Müller and Weiler 2000; Ouyang et al. 2000)</td>
</tr>
<tr>
<td>YUCCA, YUCCA2, YUCCA3</td>
<td>Arabidopsis</td>
<td>FMO-like</td>
<td>cytoplasm</td>
<td>OE: high IAA. Reduced hypocotyl growth, epinastic leaves, high number of lateral roots.</td>
<td>(Zhao et al. 2001)</td>
</tr>
</tbody>
</table>
I.E.1. Trp-dependent and Trp-independent IAA biosynthesis

Although it was long assumed that plants would synthesize IAA from Trp, early suggestions of a Trp-independent pathway came from the demonstration that indole, but not Trp, has auxin activity in an Avena coleoptile bioassay (Winter 1966). A variety of gymnosperms, angiosperms, ferns, liverworts, and mosses use both Trp-dependent and Trp-independent IAA biosynthetic pathways (Normanly et al. 1995; Sztein et al. 1995; Slovin et al. 1999). This evolutionary preservation suggests that both pathways are important.

The use of heavy isotope-labeled intermediates established the existence of the Trp-independent pathway relatively recently (Normanly et al. 1993). These studies are based on the premise that, for a linear pathway, a precursor will contain higher isotopic enrichment than its derivative will. Feeding studies with Lemna gibba indicate that Trp availability does not limit IAA biosynthesis in this system (Baldi et al. 1991). In Arabidopsis seedlings, the Trp precursor [15N]-anthranilate labels IAA more completely than Trp does, whereas [2H2]-Trp is not efficiently converted into IAA (Normanly et al. 1993); this evidence supports a Trp-independent IAA biosynthetic pathway.

Analyses of Trp biosynthetic mutants also demonstrate that IAA biosynthesis is not solely Trp-dependent. The maize orange pericarp (orp) mutant is a Trp auxotroph with defects in two Trp synthase β loci. Instead of having reduced IAA levels, as would be expected if all IAA is derived from Trp, the orp mutant contains more total (free plus conjugated) IAA than wild type (Wright et al. 1991).
Similar Arabidopsis mutants provided further support for the Trp-independent pathway. The Arabidopsis *trp3-1* and *trp2-1* mutants, which are defective in Trp synthase α and β, respectively (Last et al. 1991; Radwanski et al. 1996), have low soluble Trp levels (Müller and Weiler 2000), but accumulate amide- and ester-linked IAA conjugates (Normanly et al. 1993; Ouyang et al. 2000), suggesting that excess IAA is inactivated through conjugation. Presumably, these mutant plants maintain IAA homeostasis by increasing IAA conjugation; therefore, no phenotypic effects that can be associated with deregulated IAA homeostasis are evident.

None of the enzymes catalyzing Trp-independent IAA biosynthesis have been identified. Auxotrophic mutants constitute a classic genetic tool for elucidating biosynthetic pathways, but no mutants deficient in IAA biosynthesis have been identified, presumably because of redundancy in IAA biosynthetic pathways or lethality resulting from IAA auxotrophy. The study of auxin-supersensitive mutants that produce too much IAA may be helpful to identify some of the components of IAA biosynthetic pathways.

Trp-dependent and Trp-independent pathways are developmentally regulated, indicating a tight control of auxin homeostasis. An emerging theme in IAA biosynthesis is that plants use Trp-independent pathways for IAA maintenance, but switch to Trp-dependent pathways when high IAA levels are required (Ribnicky et al. 2001; Sztein et al. 2001). Intact Arabidopsis seedlings convert more [15N]-anthranilate into IAA than into Trp, and do not efficiently convert [3H]3-Trp into IAA (Normanly et al. 1993), indicating that Trp-independent biosynthesis is important during normal growth. In contrast, Arabidopsis shoot or root explants efficiently convert [3H]3-Trp to IAA (Müller et al. 1998; Müller and Weiler 2000), suggesting that a Trp-dependent pathway is wound-
induced. Similarly, Trp-independent IAA biosynthesis predominates in 6-day-old bean seedlings, but a Trp-dependent pathway is induced by wounding (Sztein et al. 2001).

In conclusion, the evidence indicates that plants are able to actively sense the levels of free IAA, because mutants that overproduce IAA through certain pathways shut down other IAA production pathways in response. Therefore, plants are able to regulate their metabolism to maintain specific free IAA levels, and hence regulate IAA activity.

I.E.2. The balance between IAA and glucosinolates as a control for IAA homeostasis

In contrast to the lack of knowledge about the biochemical intermediates and enzymes participating in the Trp-independent IAA biosynthetic pathway, the Trp-dependent pathway is rich with potential intermediates and branches (Figure 1-3). One important branch of the Trp-dependent IAA biosynthetic pathway leads to the formation of indole glucosinolates. The formation of indole glucosinolates from indole-3-acetaldoxime (IAOx) can occur through two pathways. One of them branches from IAOx towards indole-3-acetaldoxime N-oxide, the first committed step towards the formation of indole glucosinolates catalyzed by SUR/CYP83B1/RNT1 (Delarue et al. 1998; Barlier et al. 2000; Bak et al. 2001). The second pathway may involve formation of indole-3-acetonitrile (IAN) directly from IAOx by a yet unidentified enzyme.

The conversion of Trp to IAOx can be catalyzed by two cytochrome P450s: CYP79B2/B3. CYP79B2 was identified in a yeast screen for Arabidopsis proteins that confer resistance to 5-fluoro-indole (Hull et al. 2000). 5-Fl-indole is toxic because it is converted to 5-Fl-Trp, which inhibits anthranilate synthase and is incorporated into
proteins. *CYP79B2* expression in yeast presumably results in the conversion of 5-Fl-Trp to 5-Fl-IAOx, relieving its toxicity; overexpression in Arabidopsis leads to resistance to toxic Trp analogs (Hull et al. 2000). *CYP79B2* overexpression in Arabidopsis also leads to increased levels of indole glucosinolate (Mikkelsen et al. 2000), indole-3-acetonitrile (IAN), and IAA conjugates (J. Celenza and J. Normanly, personal communication). Like the *trp2* and *trp3* mutants, *CYP79B2*-overexpressing plants have normal free IAA levels (J. Celenza and J. Normanly, personal communication), suggesting that the excess IAA is inactivated through conjugation.

The second step for the formation of indole glucosinolates, conversion of IAOx to IAOx N-oxide, is catalyzed by the cytochrome P450 CYP83B1. The *sur2* recessive mutant, defective in this enzyme, was initially isolated based on its high auxin phenotypes that include enhanced lateral root formation (Delarue et al. 1998). A mutant in the same gene was isolated independently in a reverse-genetic screen for cytochrome P450 mutants (Winkler et al. 1998). These mutants accumulate free IAA (Delarue et al. 1998; Barlier et al. 2000), indole-3-acetaldehyde (IAAld), and IAA-Asp and IAA-Glu conjugates, but have normal IAN, IAA-Leu, and IAA-Ala levels (Barlier et al. 2000). The *sur2* mutant phenotype can be rescued by growth at low pH or on low concentrations of IAA (Barlier et al. 2000); exogenous IAA might inhibit de novo IAA biosynthesis, thus reducing the IAA accumulation caused by the *sur2* block.

**I.E.3. Auxin biosynthesis negative regulators**

Other mutants have been identified that, like *sur2*, accumulate excess IAA and exhibit enhanced IAA-related phenotypes. The study of the genes defective in these
mutants has led to the identification of important points for negative regulation of IAA biosynthesis. This thesis identified IAA-supersensitive mutants, which may be defective in negative regulators for IAA biosynthesis, like those described in this section.

1.E.3.a. Rooty and indole-3-acetaldehyde oxidase

One of the first mutants described with enhanced responses to auxin is rooty (rty) (Golparaj et al. 1996); it is a recessive lethal mutant affected in IAA homeostasis that was identified through several genetic screens as sur1 (superroot), alf1 (aberrant lateral root formation), and hls3 (hookless; Boerjan et al. 1995; Celenza et al. 1995; King et al. 1995; Lehman et al. 1996). This mutation results in such an overwhelming accumulation of IAA that not even through the observed increase in IAA conjugation can the plant maintain IAA homeostasis. The RTY gene has been cloned and is similar to a gene encoding a tyrosine aminotransferase (Golparaj et al. 1996), but the role of RTY in IAA homeostasis remains unclear. An Arabidopsis aldehyde oxidase isozyme (AAO1) specific for the conversion of IAAld to IAA accumulates in the rty mutant (Seo et al. 1998). RTY might normally act to divert an IAA precursor to a secondary metabolite (Celenza 2001). The observation that AAO1 transcription and enzymatic activity are elevated in the rty mutant (Sekimoto et al. 1998; Seo et al. 1998) indicates that RTY acts genetically as a negative regulator of AAO1 expression. It would be interesting to learn the level at which this regulation occurs, and whether AAO1 disruption results in IAA deficiency or rescues the rty high auxin phenotypes.
LE.3.b. **YUCCA and Tryptamine**

The use of novel genetic strategies has also been useful in the identification of components of the Trp biosynthetic pathway and its regulation points. This is the case for **YUCCA**, a gene identified based on its overexpression phenotype (Zhao et al. 2001). Plants overexpressing **YUCCA** accumulate free IAA and display high auxin phenotypes, including long hypocotyls in the light, hookless development in the dark, epinastic cotyledons and leaves, long petioles, auxin-independent growth in tissue culture, and increased apical dominance. **YUCCA** is an Arabidopsis flavin monooxygenase (FMO)-like enzyme that apparently catalyzes the conversion of tryptamine to N-hydroxyl-tryptamine (Zhao et al. 2001).

Tryptamine was proposed to be an IAA precursor based on its auxin activity in *Avena* coleoptile elongation assays (Winter 1966). Trp is converted to tryptamine by Trp decarboxylase (TDC), a well-studied enzyme necessary for the biosynthesis of pharmaceutically valuable monoterpenoid indole alkaloids in *Catharanthus roseus* (Facchini et al. 2000). Tryptamine accumulation may be subject to feedback inhibition, since *C. roseus TDC* transcription is down-regulated by exogenous auxin (Gooddijn et al. 1992).

The overexpression phenotype suggests that **YUCCA** is rate limiting in the tryptamine pathway. This hypothesis has not been definitively tested, as a family of **YUCCA**-like enzymes is present in Arabidopsis, and disrupting **YUCCA**, **YUCCA2**, or both does not confer any obvious phenotypes (Zhao et al. 2001). The isolation of **YUCCA** accentuates the importance of non-biased genetic approaches in understanding
IAA synthesis, since the tryptamine to N-hydroxyl-tryptamine conversion catalyzed by this enzyme was not uncovered in previous biochemical studies.

**I.F. Auxin response**

Given all the effects that auxins have in plants, it is essential for the plant to be able to precisely regulate the levels and the activity of auxins in a given tissue at a given time. This thesis studies the regulation of auxin levels and the identification of auxin-supersensitive mutants. The latter may be involved in negative regulation of auxin homeostasis or in negative regulation of the auxin response. Furthermore, the importance of maintaining auxin homeostasis is emphasized by the effects of auxin and the mechanisms for the auxin response. This section discusses what is currently known about the auxin response.

The canonical hormone response pathway, primarily based on present knowledge of mammalian hormone action, implies the presence of a hormone receptor, a signal transduction pathway, and downstream effectors. For auxin, most of the components of these pathways remain to be identified, assuming that auxin action follows the mammalian hormone general model. A putative receptor, Auxin Binding Protein (ABP1) has been identified and may mediate some auxin effects (Jones et al. 1998; Chen et al. 2001). No components of the signal transduction pathway are known, although a kinase has been implicated (Christensen et al. 2000). The downstream effectors of auxin action are better understood, including a group of auxin-induced genes and their regulation. Auxin-resistant mutants have been helpful in understanding these downstream effectors, and a representative group is listed in Table I-4.
Table I-4. Representative auxin response mutants.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Gene product</th>
<th>Known hormone effects</th>
<th>Screen used for identification</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>axr1(R)</td>
<td>Similarity to E1 ubiquitin-activating enzymes</td>
<td>Auxin, cytokinin and ethylene resistance</td>
<td>Auxin-resistant roots</td>
<td>(Estelle and Somerville, 1987; Lincoln et al., 1990)</td>
</tr>
<tr>
<td>axr2(D)</td>
<td>IAA7; an Aux/IAA auxin-induced protein</td>
<td>Auxin, ethylene and abscisic acid resistance</td>
<td>Auxin-resistant roots</td>
<td>(Knee and Hangarter, 1994; Timpte et al., 1994; Wilson et al., 1990)</td>
</tr>
<tr>
<td>axr3(D)</td>
<td>IAA17; an Aux/IAA auxin-induced protein</td>
<td>Auxin and ethylene resistance; partial phenotypic rescue by cytokinin</td>
<td>Auxin-resistant roots</td>
<td>(Leyser et al., 1996; Rouse et al., 1998)</td>
</tr>
<tr>
<td>IAA28</td>
<td>Aux/IAA auxin-induced protein</td>
<td>Auxin and ethylene resistance</td>
<td>IAA-alanine and auxin-resistant roots</td>
<td>(Rogg et al., 2001)</td>
</tr>
<tr>
<td>msg1</td>
<td>ARF7</td>
<td>Auxin and ATI* resistance</td>
<td>Auxin-induced hypocotyl curvature, non-phototrophic hypocotyl, and ATI resistant</td>
<td>(Stowe-Evans et al., 1998; Watahiki and Yamamoto, 1997)</td>
</tr>
<tr>
<td>mph4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tir5(R)</td>
<td>Protein with Leucine-rich repeats and F-box. Yeast homolog implicated in ubiquitination</td>
<td>Auxin and ATI resistance</td>
<td>ATI-resistant roots</td>
<td>(Ruegger et al., 1998)</td>
</tr>
</tbody>
</table>


The auxin binding protein, ABP1, was originally identified based on its ability to bind auxin in vitro and was postulated to function as the auxin receptor (Macdonald et al. 1991). However, the majority of ABP1 is localized to the ER, and only a small fraction can be localized to the plasma membrane (Tian et al. 1995; Shimomura et al. 1999). This localization makes it improbable that ABP1 actually functions as an auxin receptor. However, given that auxin influx and efflux carriers have been identified (see section I.C), it is possible that an auxin receptor functions in the ER. Nevertheless, ABP1 does exert auxin-mediated effects: overexpression of Arabidopsis ABP1 in tobacco or maize cells leads to auxin-dependent cell growth (Jones et al. 1998), probably by hyperpolarizing the plasma membrane (Leblanc et al. 1999). In addition, ABP1 null
mutants are lethal in Arabidopsis and antisense suppression of ABP1 in tobacco leads to loss of auxin-induced cell elongation (Chen et al. 2001). However, the downstream components of the proposed ABP1 signaling network have not been identified; hence, its proposed role as an auxin receptor awaits further proof.

Another approach to understanding hormone action is through examining effects on gene expression; this approach has led to the identification of auxin-regulated genes, the downstream effectors of the auxin response. Most of the identified auxin-regulated genes act as primary response genes because their expression is independent of de novo protein synthesis, suggesting that the hormone signal is transmitted to the nucleus through pre-existing components (Abel and Theologis 1996). A group of these auxin-regulated genes is comprised of the AUX/IAA genes, which share four highly conserved domains (Abel et al. 1995; King et al. 1995). The ARF proteins (for auxin response factor) were identified by their ability to bind the promoter region of the AUX/IAA genes (Kim et al. 1997). Most ARFs share domains III and IV of the four signature domains of Aux/IAA proteins, but in contrast to Aux/IAA proteins, the ARFs have a DNA binding domain in their amino termini (Abel and Theologis 1996). ARFs can homo- or heterodimerize to bind DNA, and Aux/IAA binding to ARF proteins inhibits ARF-mediated activation of transcription (Kim et al. 1997). The incidence of mutations in domain II of AUX/IAA proteins has implicated this domain in controlling protein stability (Nagpal et al. 2000; Rogg et al. 2001). Domain II is necessary and sufficient to confer auxin-dependent degradation to an Aux/IAA-luciferase fusion protein (Senzel et al. 2001).

Aux/IAA proteins are first rapidly induced and then rapidly degraded in the presence of auxin. The auxin-dependent degradation is dependent on the ubiquitin
pathway. The auxin-resistant 

\textit{axr1} mutant is defective in a gene with high similarity to

the amino terminal half of canonical E1 ubiquitin-ligases (Leyser et al. 1993). AXR1 and

its homologs are required in the first step leading to ligation of RUB1, an ubiquitin-like

molecule (Pozo et al. 1998). In addition, mutations in the F-box protein TIR1 result in

auxin resistance (Ruegger et al. 1998). TIR1 is part of the SCF$^{TIR1}$ E3 ubiquitin-ligase

complex formed by ASK1, AtCUL1, and TIR1; mutations in ASK1 also result in reduced

auxin responses (Gray et al. 1999). Furthermore, auxin can stimulate the interaction

between the SCF$^{TIR1}$ complex and Aux/IAA proteins leading to their degradation (Gray et

al. 2001).

The emerging picture proposes that the short-lived Aux/IAA proteins, which are

rapidly induced in response to auxin, can interfere with ARF-mediated transcriptional

activation of AuxREs, thereby altering gene expression. There are about 25 Aux/IAA

proteins and 23 ARF proteins identified in Arabidopsis (Reed 2001); this large number

suggests that the regulation of gene expression can depend on the specific Aux/IAA-ARF
dimer, whose identity is probably spatially and temporally regulated. Aux/IAA proteins
are targeted for degradation in response to auxin by binding the SCF$^{TIR1}$ E3 ubiquitin-
ligase complex. This degradation of the Aux/IAA proteins allows the ARF dimers to
induce transcription of auxin-induced genes. In the absence of efficient ubiquitination of
 Aux/IAA proteins, as in an \textit{axr1} mutant background or in the presence of stabilized
 Aux/IAA proteins, this degradation does not proceed, and the resulting plant is resistant
to auxin. The mechanisms through which Aux/IAA proteins are targeted for degradation
or for auxin-regulated degradation remain to be identified. Some mutations that stabilize
 Aux/IAA proteins result in tissue-specific auxin hypersensitivity (Rouse et al. 1998).
Although this thesis did not identify mutants defective in Aux/IAA degradation, it is possible that studying auxin-supersensitive mutants like the ones identified in this thesis would lead to the identification of the components of the auxin-dependent Aux/IAA degradation pathway or of the downstream components of auxin action.

**I.G. Mutants with increased auxin sensitivity**

In contrast to the large number of auxin-resistant mutants, very little is known about auxin supersensitive mutants such as those presented in this thesis. Few examples exist in the literature, probably because a screen for auxin-supersensitive mutants is more challenging to perform than the classic auxin-resistant screen. This section presents the known auxin supersensitive mutants *sax1, prl1, anti-AtRanBP1c,* and *ein2.* Auxin-supersensitive mutants, like auxin-resistant mutants, exhibit pleiotropic effects that are also discussed in this section.

The *sax1* mutant (supersensitive to auxin) was identified in a screen for auxin hypersensitive mutants (Ephritikhine et al. 1999A). The *sax1* mutant also exhibits hypersensitivity to abscisic acid (ABA); however, further characterization showed that its primary defect lies in the brassinosteroid biosynthetic pathway. This result confirms the complicated relations between sensitivity to the different plant hormones and implies that correct brassinosteroid function is necessary for wild-type responses to auxin and ABA.

Mutations in *PRL1* (pleiotropic regulatory locus 1) also result in auxin hypersensitivity. The pleiotropic effects of this mutation include: hypersensitivity to glucose, sucrose, cytokinin, ethylene, and ABA; enhanced anthocyanin production; short hypocotyls in the dark; and short roots in the light. Cloning of the gene interrupted by a
T-DNA in the *prl* mutant led to the identification of a nuclear protein with WD repeats, which are important for protein-protein interaction (Nemeth et al. 1998). The function of PRL1 remains to be determined, although its ability to interact with the yeast SNF1 serine/threonine protein kinase, as well as its inhibitory activity on the Arabidopsis SNF1 homologs AKIN10 and AKIN11, suggests the interesting possibility that it may be involved in signaling (Bhalerao et al. 1999).

Transgenic Arabidopsis plants expressing an antisense version of the gene coding for the Ran binding protein AtRanBP1c, exhibit a phenomenal auxin supersensitive response (Kim et al. 2001). These transgenic mutants have bigger cells and fewer lateral roots than wild type when grown without exogenous auxin. However, auxin-mediated inhibition of primary root growth can be induced with auxin concentrations 1000 times lower than those required to evoke a response in wild-type plants. Ran is a GTP-binding protein that can be found in the Ran-GDP or Ran-GTP form. These two forms are asymmetrically distributed around the nuclear pore, and it is believed that this asymmetric distribution is important for nuclear transport (Kim et al. 2001). The transgenic mutant AtRanBP1c may be supersensitive to auxin because components of the auxin transduction machinery are not able to enter or leave the nucleus adequately, which may alter their half-lives.

The classical ethylene-insensitive mutant *ein2* also exhibits hypersensitivity to IAA, particularly in shoot growth (Magidin, Monroe-Augustus and Bartel, unpublished). Paradoxically, if allowed to germinate on IAA free medium and then transferred to IAA, *ein2* exhibits IAA resistance (Magidin and Bartel, unpublished). The *ein2* mutant has also been isolated as an ABA-hypersensitive mutant with an equivalent duality in its
response: ethylene inhibits the response to ABA in germination, but it enhances the ABA response in seedling root growth (Ghassemian et al. 2000). Hence, hormone regulation and response is dependent not only on the studied tissue but also on the stage of development that is being analyzed. These are only a few of the variables that must be contemplated when studying hormone regulation and its effects in plant growth.

**I.H. Metal homeostasis**

Several lines of evidence suggest a tight relationship between metal homeostasis and phytohormone regulation: cations, including aluminum and calcium, can affect auxin transport (Hasenstein and Evans 1988). RAN1, a copper transporter, is required for the ethylene-signaling cascade to work appropriately (Hirayama et al. 1999), probably because the ethylene receptors bind copper (Rodriguez et al. 1999). EIN2, a membrane protein required for a variety of hormone responses (see above) exhibits similarity to nRAMP molecules, which have been implicated in metal transport (Alonso et al. 1999).

Some of the metals encountered by plants, such as the defined micronutrients, are necessary for plant growth. Micronutrients are required in such minuscule quantities that their identification in the early half of the 20th Century was complicated because the amount of a metal present as a contaminant in another chemical provides the amount required for plant growth. To date, the identified plant micronutrients include boron, copper, cobalt, iron, molybdenum, manganese, and zinc. These metals must be present at low concentrations, because if they are present in supraoptimal quantities they can be toxic. In addition to the required metals, plants can encounter toxic metals in their environment, such as lead, mercury, and cadmium.
Some plant species are natural hyperaccumulators of specific metals and can thus tolerate growth conditions with toxic concentrations of these metals. The majority of the hyperaccumulators identified are nickel hyperaccumulators, but hyperaccumulation of arsenic, cadmium, cobalt, selenium, and zinc has also been observed (Clemens 2001). These plant species can be useful for bioremediation (Chaney et al. 1997); therefore, the identification of mutants resistant to specific metals, like ilr2 described in this thesis, can be important for designing phytoremediation strategies.

This section discusses the three phases of metal homeostasis: transport, chelation, and sequestration; and the correlation between metal and auxin homeostasis.

I.H.1. Metal transport

The plant metal transporters that have been identified in plants are represented in Figure 1-4. Some of these have been identified by their ability to confer metal resistance to yeast strains. This is the case for IRT1, a non-specific metal transporter (Eide et al. 1996). A number of IRT1 homologues have been identified based on sequence homology, and these form the ZIP family that includes ZIP1-4, all of which confer zinc uptake to yeast cells and can also transport cadmium (Guerinot and Eide 1999). IRT2 is a newly identified member of the ZIP family that can also confer yeast the ability to transport zinc, but unlike IRT1 or ZIP1-4, it has specificity for iron and zinc (Vert et al. 2001). The ZIP proteins have five to eight predicted transmembrane domains and a metal binding site of the class HXHXH (Eng et al. 1998). It is intriguing that IAR1, a gene required for IAA-conjugate sensitivity (see above) exhibits homology to the signature
sequence of the ZIP family of metal transporters (Lasswell et al. 2000), linking IAA-conjugate homeostasis to metal homeostasis.

Another class of metal transporters has homology to Nramp proteins (Natural resistance associated macrophage proteins). AtNramp1, 3, and 4 from Arabidopsis are likely to be iron transporters since they complement a yeast mutant deficient for iron transport. AtNramp3 also transports cadmium and mutations in the gene render plants cadmium tolerant (Clemens 2001). Nramp proteins are implicated in a variety of processes, and interestingly EIN2 is also an Nramp protein (Alonso et al. 1999). The mutant ein2 has been isolated in a number of hormone-related screens as an ethylene-resistant mutant (Guzman and Ecker 1990; Alonso et al. 1999), an auxin transport inhibitor-resistant mutant (Fujita and Syono 1996), an auxin-hypersensitive mutant (Magidin and Bartel, unpublished), and an ABA--hypersensitive mutant (Ghassemian et al. 2000). It has not been shown whether EIN2 functions in metal transport, but it may be at the center of the metal-hormone web.

I.H.2. Metal chelation

Once inside the cell, metal ions are highly soluble and reactive; thus, they must be chaperoned by metal chelators. Metal chelators can be divided into four groups: methallothioneins, organic acids, amino acids, and metal chelatases.
Figure I-4. Plasma membrane and intracellular plant metal transporters. ZIP1-4, IRT1, and IRT2 are members of the ZIP family of ion transporters. AtNRAMP 1/3/4 are Nramp proteins. COPT1 is a copper transporting ATPase. Ions can enter organelles bound to PCs, through ATP-dependent transporters like CCH1, through metal antiporters like CAX1 and CAX2, or through cation diffusion facilitators like ZAT (modified from Clemens 2001).
Methallothioneins (MTs) are Cys-rich peptides that sequester metals in the cytoplasm. Methallothioneins are developmentally regulated, and their accumulation in plants can respond to a variety of stimuli ranging from metal exposure, osmotic stress, and, interestingly, hormone treatments (Rauser 1999). Three classes of MTs can be defined based on their metal-binding motifs. Class I consists of proteins that contain the canonical CC, CXC, or CXXC metal binding motifs; these have been found mainly in animals, and the Cys residues can represent as much as 30% of the protein (Rauser 1999). Class II MTs exhibit a variety of Cys distributions that are rarely homologous between them; these have been found in plants, fungi, cyanobacteria, and some arthropods (Rauser 1999). In Arabidopsis two MT-II proteins have been identified and their accumulation was found to be induced by, and bind, copper ions (Murphy et al. 1997). The third class, phytochelatins (PCs) are peptides with the general structure (γ-Glu-Cys)n-X that chelate cadmium and copper.

PC accumulation is induced in response to metals (Rauser 1995); their general structure (γ-Glu-Cys)n-X, includes PCs where X can be Gly, β-Ala, Cys, Ser or Glu (Rauser 1995; Rauser 1999). The Gly PCs are induced in response to cadmium exposure all across the plant kingdom; the β-Ala PCs have been found only in the Fabaceae family also in response to cadmium; and the Cys and Ser PCs are restricted to different graminaceous species (Rauser 1995). The biosynthesis of PCs is dependent on a γ-glutamylcysteine synthetase that joins Glu and Cys in an ATP-dependent manner. A glutathione synthetase catalyzes the addition of the Gly residue to the Gly PCs, also in an ATP-dependent reaction. Cadmium-hypersensitive mutants in Arabidopsis exhibit decreased accumulation of PCs (Howden et al. 1995), and the cadmium hypersensitive
mutant cad2 is actually deficient in a γ-glutamylcysteine synthetase, which catalyzes the first step for glutathione biosynthesis (Cobbett et al. 1998). This enzyme may catalyze the addition of the Gly residue to the Gly PCs, indicating the importance of PCs in metal tolerance (Howden and Cobbett 1992; Howden et al. 1995).

Organic acids and amino acids can also function as metal chelators. Citrate, malate and oxalate can chelate cadmium, nickel, and zinc (Rauser 1999). In response to metals, some nickel-hyperaccumulating Alyssum species exhibit an increase in their levels of His. However, this His accumulation has not been observed in other nickel-hyperaccumulators (Clercens 2001).

A number of proteins use prosthetic groups with metal cofactors. These metal ions are chaperoned by metal-specific chelatases that insert the metal into the prosthetic group. The chelatases share few sequence similarities among them, but based on their functionality they can be divided into two classes. Class 1 contains ATP-dependent heterotrimeric complexes such as the aerobic cobalamin biosynthetic cobalt chelatases CobN, CobS and CobT (Roth et al. 1996). An Arabidopsis homologue of CobN can be identified by sequence analysis, but no CobS or CobT homologues can be identified. Given that CobN is also homologous to BchlH, a chlorophyll magnesium chelatase, it seems likely that the Arabidopsis CobN homologue is in reality a BchlH homologue. Class 2 consists of ATP-independent single subunit chelatases, which include the anaerobic cobalt chelatases cbiK and cbiX of which no Arabidopsis homologues can be identified based on database analysis. However, cbiK and cbiX do not share sequence homology between them; they do, nonetheless, share a high degree of structure similarity (Schubert et al. 1999).
I.H.3. Intracellular metal sequestration

Once inside the cell, one mechanism used to control the accumulation of metals is sequestration into organelles. Several mechanisms exist for sequestering metals inside organelles. For example, PC-bound ions can be transported into the vacuole by HMT1, an ABC-type transporter that mediates cadmium resistance (Clemens 2001). The yeast protein COT1 was identified because of its ability to confer cobalt resistance when overexpressed (Conklin et al. 1992), for it induces cobalt accumulation inside organelles. COT1 is a member of the Cation Diffusion Facilitator (CDF) family, members of which have been identified in bacteria, yeast, animals, and plants (Clemens 2001). Several COT1 homologues can be identified from the Arabidopsis database, but it is not known whether they are important for cobalt transport. ZAT1 is an Arabidopsis CDF that is hypothesized to be responsible for vacuolar zinc sequestration (van der Zaal et al. 1999). In addition to CDFs, metal antiporters have been identified from plants: the Arabidopsis CAX1 and CAX2 proteins were identified by their ability to rescue a yeast strain deficient in calcium vacuolar uptake. CAX1 and CAX2 have high similarity to microbial Ca²⁺/H⁺ antiporters (Hirschi et al. 1996). Overexpression of CAX1 leads to increased calcium accumulation in tobacco (Hirschi 1999), but overexpression of CAX2 leads to calcium, cadmium, and manganese accumulation, and confers manganese resistance. This suggests that CAX2 is not specific for calcium transport (Hirschi et al. 2000).

I.H.4. Metals and auxin

Several correlations have been made between metal effects and hormones. In 1940, when the requirements for micronutrients was only being discovered, Skoog hypothesized that the effects of zinc deficiency were in part due to defects in IAA
accumulation (Skoog 1940). He found that, in contrast to copper or manganese, zinc deficiency resulted in a rapid decrease of IAA concentrations in shoots of tomato and sunflower plants. Concomitant with the reduction in IAA concentrations, zinc deficiency causes reduced chlorophyll and ABA accumulation, but addition of zinc restores the concentration of IAA within 96 hours while it does not have the same direct effect on ABA or chlorophyll (Cakmak et al. 1989).

Another case linking auxin and metals are the genes parA and parB. These genes were initially isolated as auxin-induced in tobacco cell cultures (Takahashi et al. 1995) and later as aluminum-induced in transgenic Arabidopsis lines expressing these tobacco genes (Ezaki et al. 2000). Aluminum had previously been found to increase IAA acropetal transport; however, this effect could be due indirectly to the effects of aluminum on membrane permeability (Hasenstein and Evans 1988). Upon further analysis, the parA gene, whose product is believed to function as a transcriptional activator, was found to contain a 29-bp element in its promoter. This element confers induction by IAA but not other phytohormones, and by cadmium but not copper (Kusaba et al. 1996), thus showing its specificity in linking these two elements. The parB gene codes for a glutathione-S-transferase, which confers resistance to oxidative stress, aluminum, copper, and sodium when overexpressed in Arabidopsis (Ezaki et al. 2000). It is attractive to speculate that, like CAD2, PARB could function in PC synthesis linking auxin and metal tolerance.

Furthermore, several proteins known to be important for auxin homeostasis are also dependent on metal cofactors (see above: ILR1, IAR3, and AOX). The availability of these cofactors may also be a regulatory mechanism for auxin homeostasis.
This thesis presents the identification of *ILR2*, a gene important for IAA-Leu sensitivity, and the characterization of the *ilr2* mutant. The *ilr2* mutant is resistant to the effects of cobalt and manganese, thus suggesting yet another link between auxin homeostasis and metal metabolism.
CHAPTER II. MATERIALS AND METHODS

II.A. Plant materials and growth conditions

I used *Arabidopsis thaliana* for all of the analyses presented in this thesis. For phenotypic studies, the seeds of the specified ecotypes (Table II-1) and genotypes were surface-sterilized with 20% bleach and 0.01% Triton X-100 for 12 minutes, followed by two washes with water, and left in 0.1% agar solution at 4 °C for 24-48 hours prior to plating on plant nutrient medium (PN; Haughn and Somerville 1986). PN was supplemented with 0.6% agar, except for root-waving experiments, where it was supplemented with 1.5% agar.

For phenotypic analyses of hormone effects, including IAA and IAA conjugates, PN was supplemented with 0.5% sucrose (PNS), except where indicated. Hormone-supplemented media was prepared using ethanol-based stocks. Hormones were from Aldrich (Milwaukee, WI), except for IAA-*myo*-inositol and JA-Phe, which were synthesized by Kristin Krukenberg and Seiichi Matsuda. For phenotypic analysis of metal effects, PN was not supplemented with sucrose, and it was prepared using filter-sterilized water-based stocks. For antibiotic selection, I used 15 μg/mL kanamycin or 7.5 μg/mL glufosinate-ammonium (BASTA).

For growth on PN, seedlings were grown on plates sealed with gas-permeable surgical tape (LecTec Corp., Minnetonka, MN), and grown at 22 °C with continuous illumination (25 to 45 μE m⁻² sec⁻¹) under yellow long-pass filters that slow breakdown of
indolic compounds (Stasinopoulos and Hangarter 1990). For high temperature experiments, plants were grown under the same conditions at 28 °C. The yellow light filters were used for all analysis of hormone and metal effects on root growth to allow meaningful comparisons.

For growth in soil, seedlings were either transferred from plates or seeds were sown directly in soil (Metromix 200; Scotts, Marysville OH), and grown at 22 to 25 °C under continuous light or 8 hour light/16 hour dark cycles, using Cool White fluorescent bulbs (≈ 200 μE m⁻² sec⁻¹; Sylvania, Versailles, KY).

<table>
<thead>
<tr>
<th>Ecotype name</th>
<th>Origin</th>
<th>ABRC Stock number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Columbia (Col-0)</td>
<td>Columbia, MO, USA</td>
<td>CS1092</td>
</tr>
<tr>
<td>Landsberg erecta tt4 (Ler)</td>
<td>Landsberg, Germany</td>
<td>CS85</td>
</tr>
<tr>
<td>Wassilewskija (Ws)</td>
<td>Wassilewskija, Russia</td>
<td>CS1602</td>
</tr>
<tr>
<td>Mr-0</td>
<td>Monte/Tosso, Italy</td>
<td>CS1372</td>
</tr>
<tr>
<td>Pog-0</td>
<td>Point Grey, Canada</td>
<td>CS1476</td>
</tr>
<tr>
<td>Uk-2</td>
<td>Umkirch, Germany</td>
<td>CS1578</td>
</tr>
<tr>
<td>Bla-10</td>
<td>Blanes/Gerona, Spain</td>
<td>CS982</td>
</tr>
<tr>
<td>Yo-0</td>
<td>Yosemite/CA, USA</td>
<td>CS1622</td>
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<tr>
<td>Bl-1</td>
<td>Bologna, Italy</td>
<td>CS968</td>
</tr>
<tr>
<td>XXX-0</td>
<td>N/A</td>
<td>CS1620</td>
</tr>
<tr>
<td>Mt-0</td>
<td>Martuba/Cyrenaika, Libya</td>
<td>CS1380</td>
</tr>
<tr>
<td>Tsu-0</td>
<td>Tsu, Japan</td>
<td>CS1564</td>
</tr>
<tr>
<td>Es-0</td>
<td>Espoo, Finland</td>
<td>CS1144</td>
</tr>
</tbody>
</table>

II.B. *ilr2* mutant isolation

The *ilr2-1* mutant was isolated in an IAA-Leu resistance screen (Bartel and Fink 1995) from the M₁ progeny of Ws seed mutagenized with ethyl methanesulfonate (EMS). M₂ seeds were spread on PNS containing 40 μM IAA-Leu and after 10 days putative mutants with longer than wild-type roots were selected. These plants were transferred to soil and allowed to set seed, and the resultant M₃ seedlings were assayed for IAA-Leu
resistant plants that responded normally to IAA. The \textit{ilr2-1} mutant was backcrossed three times to the parental Ws line prior to phenotypic analysis.

The Col-0 \textit{ilr2-2} allele was kindly supplied by the Torrey Mesa Research Institute (San Diego, CA). This allele was identified from a collection of individual T-DNA mutagenized Col-0 plants, in which the T-DNA insertion point was identified by sequencing DNA flanking the left border of the T-DNA. These sequences are available in a BLAST-searchable database (www.nadii.com/pages/collaborations/garlic_files), which I searched using the \textit{ILR2} sequence to identify the only T-DNA insertional mutant in \textit{ILR2}. The T-DNA in \textit{ilr2-2} is inserted 140 bp upstream from the first ATG of the predicted Col-0 \textit{ILR2} transcript. The location of this T-DNA was verified by PCR using the T-DNA left border specific primer LBSYN-1 and the \textit{ILR2} specific primer MYF-68 (Table II-2).
<table>
<thead>
<tr>
<th>Table II-2. Oligonucleotides used in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td>7G6-3</td>
</tr>
<tr>
<td>7G6-4</td>
</tr>
<tr>
<td>F130I1-1</td>
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<tr>
<td>MEB5-R1</td>
</tr>
<tr>
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<td>MIE15-2</td>
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</tr>
<tr>
<td>MYF24-62</td>
</tr>
<tr>
<td>MYF24-68</td>
</tr>
<tr>
<td>RILR2-MSma*</td>
</tr>
<tr>
<td>RILR2-MWS*</td>
</tr>
<tr>
<td>T9L6-1</td>
</tr>
<tr>
<td>T9L6-2</td>
</tr>
</tbody>
</table>

Sequences are 5'-3'. In the oligonucleotides RILR2-MSma and RILR2-MWS used for oligo-directed mutagenesis, lower case nucleotides indicate the changes introduced; in RILR2-MWS: the bolded bases indicate the position of the spanned Ws deletion.
II.C. *axes* mutant screen

The *axes* screen was performed using the M$_2$ progeny of Col-0 seed mutagenized with ethyl methanesulfonate (EMS). M$_2$ seeds were plated individually in rows on sterilized cellophane membranes placed on PNS plates. After 4-5 days of growth, the cellophane membranes were transferred to media supplemented with 20 nM IAA, and the plate was turned 180° to alter the direction of root growth. Four to five days later, putative mutants with roots shorter than wild type were selected. These plants were transferred to soil, allowed to set seed, and resultant M$_3$ seedlings were assayed in a secondary screen. For the secondary screen, M$_3$ seeds were plated on PNS with or without 25 nM IAA. The primary root elongation of the seedlings was measured after 8 days of growth to identify M$_3$ seedlings that exhibited auxin supersensitivity. Then, the M$_3$ seeds, or when necessary the M$_4$ seeds, from the selected lines were analyzed in a tertiary screen to analyze the effect of an auxin gradient on root growth and on lateral root formation. For root-growth analysis, seeds were plated on unsupplemented PNS or on PNS media supplemented with a gradient of IAA concentrations ranging from 1 to 100 nM. To analyze the effect of auxins on lateral root formation, seeds were plated on PNS medium where they grew for 4 days prior to their transfer to PNS media supplemented with 5 or 10 μM IBA or with 5 or 10 nM IAA. Four days after transfer, the number of lateral roots was counted using a dissecting microscope. The mutants selected as a result of the tertiary screen were back-crossed to the parental wild type (Col-0) and out-crossed to Ws or Ler wild type for mapping.
II.D. *axs* and *ilr2* mutant characterization

Root elongation in the presence of hormones was assayed using one of two methods, as indicated in each experiment. The first method involved sowing seeds directly on medium supplemented with the indicated compound or in the indicated temperature; after 8 to 13 days the seedlings were removed from the medium and the lengths of the primary roots measured. The second method used to measure root elongation was to allow seedlings to grow on unsupplemented medium for 4-5 days prior to transferring the seedlings to media supplemented with the indicated compounds. The seedlings were aligned on the new plates and the plates were inverted 180°, allowing the roots to turn in response to the gravity vector and grow in a different direction from the aligned axis. After the indicated number of days, the root growth that occurred after transfer was measured.

Lateral root promotion was assayed using similar methods. However, for lateral roots, the total number of lateral roots present on each seedling was counted even in experiments involving seedling transfer. Lateral roots were counted using a dissecting microscope; any visible primordia that had emerged from the primary root were counted.

For the *ilr2-1* root growth experiment, seeds were sown on PNS or on PNS containing 20 μM IAA-Leu, and the plates were placed vertically to allow the roots to extend downward over the surface of the plates. At the indicated times, the position of the root tip was marked on the plates and subsequently the distance in between marks was measured.
The hypocotyl experiments were performed by plating the seeds on PNS media and measuring the length of the hypocotyl after 8 days of growth at 22 or 28 °C.

For germination experiments, seeds were placed at 4 °C for 48 hours in 0.1% agar prior to plating on PNS medium. Using a dissecting microscope, the seeds were analyzed at specified intervals for radicle emergence.

Root waving experiments were performed as described (Fujita and Syono 1997). Seeds were plated on medium containing 1.5% agar and grown under yellow light in a horizontal position. After 3 days, the plates were positioned at a 30° angle and the seedlings were allowed to grow for 5 additional days before analyzing the root-waving patterns.

To assay the ethylene triple response in axs mutants, seeds were plated on unsupplemented PNS or on PNS supplemented with 10 μM 1-aminocyclopropane-1-carboxylic acid (ACC). The plates were incubated under yellow light at 22 °C for 24 hours. The plates were then covered with foil and the seedlings grew in the dark for 7 additional days. The seedlings were examined for ACC-mediated inhibition of root and hypocotyl elongation in the dark.

**II.F. Genetic mapping of ilr2 and axs mutations**

The mutants were mapped using two types of PCR-based polymorphisms: Simple Sequence Length Polymorphisms (SSLP; Bell and Ecker 1994) or Cleaved Amplified Polymorphic Sequences (CAPS; Konieczny and Ausubel 1993). SSLP markers are PCR-amplified sequences that have distinct sizes in the different ecotypes. CAPS markers
consist on PCR-amplified sequences that have altered restriction digest patterns in the different ecotypes.

Mapping was initially done using previously described SSLP or CAPS markers (www.arabidopsis.org) to identify the chromosomal linkage of the analyzed mutations, and additional markers were developed as needed. The new PCR markers were developed by sequencing PCR-amplified Ler or Ws sequences and comparing these to the publicly available Col-0 sequence (Kaul et al. 2000) to identify SSLP or CAPS markers. Alternatively, regions with Single Nucleotide Polymorphisms identified for Col-0/Ler DNA (http://arabidopsis.org/SNPs.html) were PCR-amplified and tested in Col-0, Ler, and Ws. The new markers are listed in Table IV-1 and Table V-2.

II.F. ilr2 mapping

The ilr2-1 mutation was mapped using segregating F2 populations from crosses between ilr2-1 mutant (Ws background) and wild-type Col-0 or Ler plants. Approximately 3000 F2 seeds were plated on PNS supplemented with 40 μM IAA-Leu and genomic DNA was prepared (Celenza et al. 1995) from approximately 700 IAA-Leu resistant plants. The progeny from IAA-Leu resistant plants that appeared heterozygous at the borders of the mapping region were screened for IAA-Leu resistance to confirm their ilr2/ilr2 genotype.

II.G. axs mapping

The axs1 and axs2 mutants were mapped using segregating F2 populations from crosses between axs1 (Col-0 background) and wild-type Ws, or between axs2 (Col-0
background) and wild-type Ler. F₂ seeds were plated on PNS and genomic DNA was prepared (Celenza et al. 1995) from plants that exhibited more relaxed root curling than wild-type Ler or Ws seedlings. The axs3 mutant was mapped using a segregating F₂ population from a cross between axs3 (Col-0 background) and wild-type Ler. F₂ seeds were plated on PNS at 28 °C and seedlings with short roots were selected and transferred to PNS at 22 °C for 3-5 days before transferring to soil. Genomic DNA was prepared from these plants as described (Celenza et al. 1995).

II.H. Complementation cloning of ilr2

DNA from BAC T10F24 (obtained from the Arabidopsis Biological Resource Center; ABRC) was obtained by growing three large scale 600 mL bacteria cultures for 15 hours and purifying DNA using JetStar (Genomed, Germany) anion exchange columns. Approximately 50 μg of DNA were partially digested with Sau3AI (NEB) for 25 minutes (previously determined to produce a majority of 10-12 kb fragments). The digest was electrophoresed on 0.8% agarose supplemented with 1 mM guanosine (Gründemann and Schömig 1996) and 10-12 kb fragments were purified using JetSorb resin (Genomed, Germany), following the manufacturer’s recommendations. The fragments were ligated into the BamHI site of the binary vector pBIN19 (Bevan 1984) and transformed into Escherichia coli DH5α. The clones were ordered in a contig based on the sequences from the P1 clones MYF24 and MIE15, which are contained in the T10F24 BAC (Kaul et al. 2000), using DNA dot hybridization at foci in the MYF24 area and restriction digest analysis. The P1 clone sequence was analyzed using Genscan and Grail software (Burge and Karlin 1997; Hyatt et al. 2000) to identify the putative open
reading frames (ORFs), thus enabling the complete coverage of ORFs by the library. ORFs predicted in MIE15 that were not contained in the T10F24 BAC, were sequenced from the ilr2-1 mutant following PCR amplification of genomic DNA prepared as described previously (Celenza et al. 1995). Amplified PCR products were purified by sequential ethanol, polyethylene glycol, and ethanol precipitations (Ausubel et al. 1995), and sequenced using an automated DNA sequencer (Lone Star Laboratories, Inc., Houston, TX) using the same primers as for the PCR amplification.

Overlapping clones from T10F24 were transformed into Agrobacterium tumefaciens GV3101 (Koncz and Schell 1986) by electroporation (Ausubel et al. 1995). ilr2-1 mutant plants were transformed with these constructs using the floral dip method as previously described (Desfeux et al. 1998). Seeds from these plants were germinated on PN supplemented with 15 μg/mL kanamycin to identify T1 plants with inserted DNA. The T2 progeny of these plants was tested for restoration of wild-type sensitivity to IAA-Leu. The ILR2 gene was sequenced from the ilr2 mutant genomic DNA following PCR amplification. Primers used were MYF24-60 together with MYF24-57 (Table II-2), which yields an ~1120-bp Col-0 product and a 120-bp product in Ws and ilr2-1 and MYF24-62 with MYF24-61 (Table II-2), which yields a 990 bp product in both Col-0 and Ws.

II.I. RT-PCR and 5’-RACE

RNA was extracted from 8-day-old Col-0 and Ws seedlings grown on PNS plates covered with filters using Trizol (Gibco) following the manufacturer’s instructions, or using the LiCl method which uses aurin tricarboxylic acid as previously described (Nagy
et al. 1988). The RNA was electrophoresed to ascertain its integrity. The RNA extracted
with aurin tricarboxylic acid was purified to remove chemical contaminants using
miniQuick spin columns (Boehringer Mannheim, Indianapolis).

RT-PCR and 5’-RACE PCR were carried out using Ambion (Austin, TX) RETROScript and RLM-RACE kits following the manufacturer’s instructions. For 5’-RACE the gene specific outer primer used was MYF24-59 (Table II-2), and the gene specific inner primer used was MYF24-57 (Table II-2).

**II.1. RNA and Northern Blot analysis**

Total RNA was prepared as described (Nagy et al. 1988) from whole seedlings
grown on PNS, supplemented as indicated, and overlaid with 3MW gel blot paper
(Midwest Scientific); or from leaves, stems, and flowers from plants grown in soil for 6
weeks in 8/16 hour light/dark cycles and then for 6 weeks in continuous light. For metal
exposure, seedlings were grown for 8 days on PNS and then transferred for 17 hours to
unsupplemented PN media, or to PN media supplemented with 0.1 mM CuSO$_4$, 5 mM
MnCl$_2$, 1 mM ZnSO$_4$, 1 mM CoCl$_2$, or 1 mM Fe-EDTA.

Approximately 5-10 $\mu$g of total RNA was separated on a 1% agarose gel containing
0.37 M formaldehyde and transferred to Bright-Star Plus nylon membranes (Ambion,
Austin TX). The *ILR2* probe, a 605 bp *BglII* fragment from pKSColILR2 (Table II-3),
was $^{32}$P-labeled using random 12-mer oligonucleotides as primers (Ausubel et al. 1999).
After prehybridizing in ULTRAhyb Ultrasensitive Hybridization Buffer (Ambion;
Austin, TX), the probe was hybridized overnight at 65 °C and washed according to the
manufacturer’s instructions.
II.K. Plasmid construction and transgenic lines

The plasmids constructed for this study are listed in Table II-3. All the plant transformations were performed by first transforming the plasmids into Agrobacterium tumefaciens GV3101 (Koncz and Schell 1986) by electroporation (Ausubel et al. 1995). Then, the plants of the indicated genotypes were transformed with the described constructs using the floral dip method (Clough and Bent 1998), modified as described by Desfeux et al. (1998).

II.K.1. Expression of genomic copies of short and long ILR2

The 5.9 kb EcoRI fragment containing the Col-0 version of ILR2 was subcloned from BAC T10F24 (ABRC) into EcoRI-cut pBluescript KS (+) to give pKSCollLR2, and into EcoRI-cut pBIN19 (Bevan 1984) to give pBIRILR2. The Ws version of ILR2 was obtained by removing 983 bp from pKSCollLR2 using oligonucleotide-directed mutagenesis (Ausubel et al. 1999) with RILR2-Mws (Table II-2), to give pKSWILR2. The 5.4 kb PvuII fragment from pKSWILR2 was subcloned into the pBIN19 SmaI site to give pBWILR2. pBIRILR2 and pBWILR2 were transformed into ilr2-1 mutant plants as well as into wild-type Col-0 and Ws plants. Transformants were selected on PN supplemented with kanamycin (T1 seedlings), and their descendents were analyzed for rescue of the ilr2-1 IAA-Leu and cobalt resistance.
<table>
<thead>
<tr>
<th>Plasmid name</th>
<th>Purpose</th>
<th>Description</th>
<th>Strain number</th>
</tr>
</thead>
<tbody>
<tr>
<td>35SCLIR2</td>
<td>long ILR2 genomic overexpression</td>
<td>BsrBI fragment from pKCILR2.2 subcloned into 35SpBARN</td>
<td>1196, 1197</td>
</tr>
<tr>
<td>CaMVILR2myc</td>
<td>overexpression of myc tagged long ILR2</td>
<td>Bsal-PvuII fragment from pKRILR2myc subcloned into 35S CaMV</td>
<td>1194, 1195</td>
</tr>
<tr>
<td>pBINILR2myc</td>
<td>expression of myc tagged long ILR2</td>
<td>PvuII fragment from pKRILR2myc subcloned into pBIN19</td>
<td>1165, 1169</td>
</tr>
<tr>
<td>pBINWILR2myc</td>
<td>expression of myc tagged short ILR2</td>
<td>PvuII fragment from pKWSILR2myc subcloned into pBIN19</td>
<td>1198, 1199</td>
</tr>
<tr>
<td>pBIRILR2</td>
<td>ilr2 complementation</td>
<td>EcoRI fragment from pKScollIR2 subcloned into pBIN19</td>
<td>1192, 1193</td>
</tr>
<tr>
<td>pBWILR2</td>
<td>short ILR2 for complementation</td>
<td>PvuII fragment from pKSWILR2 subcloned into pBIN19</td>
<td>1200, 1201</td>
</tr>
<tr>
<td>pKCILR2</td>
<td>precursor</td>
<td>DraI-SmaI fragment from pKScollILR2 subcloned into SmaI site of Bluescript KS(-)</td>
<td>1136</td>
</tr>
<tr>
<td>pKCILR2.2</td>
<td>precursor for long ILR2 genomic overexpression</td>
<td>NdeI-SmaI fragment from pKRILR2 blunted and subcloned into Bluescript KS(-)</td>
<td>1136</td>
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<tr>
<td>pKRILR2myc</td>
<td>precursor for myc tagged version of long ILR2</td>
<td>SmaI fragment removed from pKRILR2Sma, and five tandem copies of the myc epitope inserted into SmaI site</td>
<td>1131</td>
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<tr>
<td>pKScollIR2</td>
<td>precursor</td>
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<tr>
<td>pKSmallIRL2</td>
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<td>NdeI-EcoNI dropped out of pKScollILR2</td>
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<td>pKScollIR2 mutagenized with ILR2-MSma</td>
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<tr>
<td>pKWSILR2myc</td>
<td>precursor for myc tagged version of short ILR2</td>
<td>SmaI fragment removed from pKWSILR2Sma, and five tandem copies of myc inserted into SmaI site</td>
<td>1191</td>
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<tr>
<td>pKWSILR2Sma</td>
<td>precursor for myc tagged version of short ILR2</td>
<td>Combined XbaI-Stul fragment from pKSRLR2Sma (containing Sma mutagenesis) with Xba-Stul fragment from pKWSILR2 (containing Ws mutagenesis)</td>
<td>1125</td>
</tr>
</tbody>
</table>

Constructs with two strain numbers include the number for the *E. coli* strain carrying the plasmid and for the *Agrobacterium* strain carrying the same plasmid.
II.K.2. Epitope-tagged genomic copies of short and long ILR2

For the epitope tagged constructs, pKSCollLR2 and pKSWILR2 were mutagenized using oligonucleotide-directed mutageneses (Ausubel et al. 1999) with RILR2-MSma (Table II-2), which destroys the stop codon by transforming it into a SmaI site, to give pKSRILR2Sma and pKWSILR2Sma, respectively. Then, a Dral-SmaI fragment containing five tandem copies of the myc epitope (Jarvik and Telmer 1998) was inserted into the SmaI site of pKILR2Sma and pKWILR2Sma, to give pKILR2myc and pKWILR2myc. PvuII fragments from the resultant plasmids, which contain the ILR2myc fusions and ~2000 bp upstream sequence, were ligated into the EcoRI site of pBIN19, to give pBILR2myc and pBIWILR2myc. These plasmids were transformed into Col-0, Ws, and ilr2-1; transformants were selected by plating on PN supplemented with kanamycin as described.

II.K.3. Overexpression of ILR2

Overexpression of ILR2 was achieved by cloning the predicted Col-0 ORF into one of two vectors: 35SpBARN (LeClere and Bartel 2001) or 35SCaMV (Hull et al. 2000). Both vectors contain the Cauliflower Mosaic Virus (CaMV) 35S strong promoter to drive overexpression of the genes. To overexpress the Col-0 form of ILR2, the ~4 Kb Dral/SmaI fragment of pKSCollLR2 was ligated into the SmaI site of pBluescript KS (+) to give pKCIILR2.2. The BsrBI fragment from the resultant plasmid was ligated into the SmaI-cut 35SpBARN (LeClere and Bartel 2001), to give p35SCILR2. To overexpress the myc tagged Col ILR2, the ~4.4 Kb PvuII fragment from pKILR2myc was subcloned into the SmaI site of the 35SCaMV vector (Hull et al. 2000), to give 35SCamVILR2myc. Plasmids 35SCILR2 and 35SCaMVILR2myc were transformed into Col-0, Ws, and
*ilr2-1* plants. Transformants with the 35SpBARN-derived plasmids were selected by plating on BASTA; transformants with the 35SCaMV-derived plasmids were selected by plating on kanamycin.

**III. Atomic absorption**

Surface sterilized seeds were plated on 3MW Gel Blot Paper (Midwest Scientific) placed on PN media supplemented as indicated. Approximately 150 seeds were plated on each plate, and three plates were used for each experimental repetition. The plants were allowed to grow for 10 days prior to harvesting; the tissue was then placed in a 70-80 °C oven for approximately 7-10 days until the tissue was completely dry. The dried tissue was ground to a fine powder using a mortar and pestle. After weighing the dry tissue, it was sent to the Department of Fruit and Vegetable Sciences at Cornell University for atomic absorption analysis of metal content using the dry ash method.
CHAPTER III. PHENOTYPIC CHARACTERIZATION OF \textit{ilr2-1}

The conjugation and deconjugation of IAA to small molecules such as amino acids or peptides is important for maintaining IAA homeostasis. To further understand the role and regulation of IAA conjugation and deconjugation, we are characterizing mutants that are resistant to conjugated forms of the hormone, but that remain sensitive to free IAA. These mutants genetically separate the effects of IAA from those of IAA conjugates; therefore, their study allows the identification of the roles that IAA conjugates play and the processes important for IAA deconjugation. This chapter describes the phenotypic characterization of \textit{ilr2-1}, a novel IAA-conjugate resistant mutant. The mutant \textit{ilr2-1} was isolated by B. Bartel as IAA-Leu resistant, but sensitive to free IAA.

\textbf{III.A. The \textit{ilr2-1} mutant is resistant to both IAA-Leu and IAA-Phe}

As a first step towards analyzing the \textit{ilr2-1} mutant, I analyzed its resistance to other forms of IAA. The \textit{ilr2-1} mutant was isolated in a genetic screen based on its IAA-Leu resistance and IAA sensitivity (Figure III-1A). To further characterize the auxin conjugate resistance profile of \textit{ilr2-1}, I grew \textit{ilr2-1} seeds in the presence of three conjugates: IAA-Ala, IAA-Leu, and IAA-Phe. These are conjugates that have been identified from Arabidopsis (IAA-Ala and IAA-Leu) and that exhibit activity in an Arabidopsis auxin bioassay (Bartel and Fink 1995; Barlier et al. 2000; Kowalczyk and Sandberg 2001). The results, illustrated in Figure III-1B, show that \textit{ilr2-1} has reduced sensitivity to IAA-Leu and IAA-Phe, but wild-type sensitivity to the free form of the hormone as well as to IAA-Ala. In conclusion, these results indicate that the different
IAA-amino acid conjugates are not equivalent. A mutation in ILR2 affects the response to IAA-Leu and IAA-Phe, but has no effect on the response to IAA-Ala.

In addition, I characterized the response of ilr2-1 and other IAA-amino acid resistant mutants to the ester conjugate IAA-myoinositol; the results are shown in Figure III-2. Most of the tested IAA-amino acid resistant mutants, including ilr2-1, respond like wild-type to this ester conjugate. This result suggests that the mechanisms regulating amide- and ester-IAA conjugation and deconjugation are different from one another. However, it is interesting that the double amidohydrolase mutant ilr1 iar3 is resistant to low concentrations of IAA-myoinositol. Given that these amidohydrolases do not cleave ester linkages (Bartel and Fink 1995), this resistance might be due to indirect effects resulting from altered conjugate homeostasis. These results indicate that amide-linked IAA conjugates and ester-linked IAA conjugates are not recognized through the same pathways as certain IAA-amino acid resistant mutants are not resistant to IAA-ester conjugates.
Figure III-1. Roots of *iir2-1* are resistant to IAA-Leu and IAA-Phe. (A) *iir2-1* mutant and Wassilewskija (Ws) wild-type seedlings grown on media containing no hormone, 100 nM IAA, or 40 μM IAA-Leu for eight days were removed from the media and photographed. (B) Primary root lengths of eight-day-old wild-type Ws and *iir2-1* mutant seedlings grown on the indicated concentrations of auxins were measured. Error bars represent the standard errors of the means (n ≥ 10).
Figure III-2. Mutants resistant to IAA-amino acids exhibit a wild-type response to the ester linked conjugate IAA-myoinositol. Primary root lengths of 8-day-old wild-type Ws, ilr2-1, iar1-1, ilr1-1 iar3-2 double amidohydrolase mutant, and iar4 seedlings grown on the indicated concentrations of auxin. Error bars represent the standard errors of the means ($n \geq 11$).
III.B. The *ilr2-1* mutant has a slower growing root than wild type

The roots of *ilr2-1* mutant seedlings exhibit auxin-related phenotypes even without hormones in the growth medium. For example, the primary root of 8-day-old *ilr2-1* seedlings is consistently shorter than wild type (Figure III-1B). To determine whether this short root reflects a delay in germination and/or a reduced elongation rate, I analyzed the root growth rate of wild type and *ilr2-1* seedlings in the absence or presence of IAA-Leu over 10 days. As shown in Figure III-3A, *ilr2-1* mutant roots grow more slowly than wild type in the absence of hormone. In the presence of IAA-Leu, the growth rate of both mutant and wild-type roots is inhibited, but *ilr2-1* is less sensitive to this inhibition than wild type (Figure III-3A). The root growth defect is not rescued by growth at 28 °C (Figure III-3B), which promotes IAA accumulation (Gray et al. 1998).
Figure III-3. The *ilr2-1* mutant has a slower growing root than wild type. *ilr2-1* mutant and Ws wild-type seeds were sown on media containing no hormone or 20 μM IAA-Leu and grown at (A) 22 °C or (B) 28 °C on vertically oriented plates. At the specified times, the length of the primary root was recorded. Error bars represent the standard errors of the means (*n* ≥ 8).
III.C. *ilr2-1* is resistant to IAA-Leu at 22 °C and 28 °C

The *ilr2-1* mutant exhibits slower root-growth than wild-type at low and high temperatures (Figure III-3). This prompted me to analyze the resistance of *ilr2-1* to a variety of concentrations of IAA-Leu at both temperatures. The results, shown in Figure III-4, indicate that the effects of IAA-Leu on wild type can be observed at both temperatures, and that *ilr2-1* is resistant to the conjugate at both temperatures as well. However, at 28 °C IAA-Leu appears more inhibitory to root elongation than at 22 °C for both wild type and *ilr2-1* mutant seedlings. Given that auxin accumulates at high temperatures (Gray et al. 1998), it is tempting to speculate that lower amounts of IAA-Leu are needed at 28 °C than at 22 °C to make the total auxin concentration in the seedling go from inductive to inhibitory for growth.
Figure III-4. The ilr2-1 mutant is resistant to IAA-Leu at 22 °C and 28 °C. Wild-type Ws and ilr2-1 mutant seedlings were grown (A) at 22 °C or (B) at 28 °C on media supplemented with the indicated concentrations of IAA-Leu. After eight days of growth, the primary roots were measured. Error bars represent standard errors of the means (n ≥ 9).
III.D. Analysis of ilr2-1 germination

The short roots of ilr2-1 could be due solely to slower root growth or to a combination of slower growth with delayed germination. I determined that ilr2-1 roots exhibit a slower growth rate (Figure III-3) but I was still interested in analyzing the germination rate. I used two approaches to analyze germination. One approach involved the analysis of an F2 population from an ilr2-1 out-cross to wild-type Col-0, where the ilr2-1 mutation was segregating. In the second approach, I analyzed the germination rate of ilr2-1 compared to that of its parental Ws ecotype, wild-type Ws.

For the segregation analysis, approximately 150 seeds segregating Col-0 (wt) and Ws (ilr2-1) genetic material were scored at different times after sowing (27, 41, 43, 48 and 52 hours) to identify the germination time of each seed. At the conclusion of the experiment, the genotypes of all the seedlings that were first to germinate, at 41 hours, and of those that were last to germinate, at 52 hours, were scored using a PCR polymorphism closely linked to the ilr2-1 mutation (MYF24, see Chapter IV). The results, shown in Table III-1, indicate that in this type of out-cross the ilr2-1 mutation does not exert any effect on germination rate. At the time points analyzed the distribution of Ws (ilr2-1) MYF24 loci was equivalent to that of Col-0 (wt) MYF24 loci.

Table III-1. The ilr2-1 mutation does not have an effect on germination rates in an F2 population from a Ws (ilr2-1) to Col-0 (wt) cross.

<table>
<thead>
<tr>
<th>Analyzed seedlings</th>
<th>Ws (ilr2-1) at MYF24</th>
<th>Heterozygote at MYF24</th>
<th>Col-0 (wt) at MYF24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germinated at 41 hours</td>
<td>3</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Germinated at 52 hours</td>
<td>1</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>

The germination time of approximately 150 seeds from an F2 population segregating Col-0 (wt) and Ws (ilr2-1) genetic material was analyzed. A PCR-based marker closely linked to the ilr2-1 mutation was analyzed from the first 12 seeds to germinate, at 41 hours, and from the last 8 seeds to germinate, at 52 hours.
The previous experiment only indicated that when the Ws and Col-0 ecotypes are mixed, the *ilr2-1* mutation does not have a definite effect on germination rate. I was still interested in determining whether *ilr2-1* had a germination defect compared to its parental ecotype, Ws. Towards this end, I compared the germination rate of *ilr2-1* seeds to that of Ws seeds. I used two populations of Ws seeds that were harvested one year before and one year after the harvest date for the *ilr2-1* seeds to account for the effect that seed age might have on germination. The results shown in Figure III-5, indicate that *ilr2-1* germinates more slowly than its parental wild type ecotype Ws. The double amidohydrolase mutant *ilr1 iar3* may also exhibit a somewhat slower germination rate than wild type, but this defect is less severe than the *ilr2-1* defect.

The apparently paradoxical results obtained with these two experiments may be explained by differences between the ecotypes that are important for overcoming the *ilr2-1* defect. In other words, quantitative trait loci (QTLs) present in the Col-0 ecotype, but not in Ws, may overcome the *ilr2-1* germination defect. These QTLs are probably specific for the germination aspects affected by *ilr2-1*, because in an F2 population from a cross between *ilr2-1* and wild-type Col-0, the IAA-Leu resistance phenotype of *ilr2-1* segregates in a ratio close to 1:3 (data not shown).
Figure III-5. Mutant *ilr2-1* seeds germinate more slowly than wild-type *Ws* seeds. Two populations of *Ws* seeds that were harvested a year prior (*Ws-1*) or a year after (*Ws-2*) the *ilr2-1* seeds were harvested, were analyzed with *ilr1* and the double amidohydrolase mutant *ilr1 iar3* at the indicated time after seed sowing. Error bars represent standard errors from the mean obtained from three experimental repetitions.

**III.E. *ilr2-1* has a fewer lateral roots.**

In addition to its root-growth defect, the *ilr2-1* mutant is also defective in lateral root formation, another auxin-related phenotype. The number of lateral roots present in *ilr2-1* mutant seedlings is lower than wild type. This phenotype is present at 22 °C, and the difference between mutant and wild type is also apparent following growth at 28 °C (Figure III-6A), a condition that promotes IAA accumulation, hypocotyl elongation (Gray et al. 1998), root elongation, and lateral root formation (Rogg et al. 2001). The *ilr2-1* defect is enhanced when crossed to *iar3*, an IAA-Ala resistant amidohydrolase mutant.
(Davies et al. 1999), suggesting an important role for auxin conjugates in secondary root induction at high temperatures. It is interesting that the other IAA-amino acid resistant mutants do not exhibit a significant defect in lateral root formation or in any other high-temperature induced phenotypes. This suggests that \textit{ilr2-1} has a low number of lateral roots because it is defective in a specific process that is important for lateral root formation, and this defect in lateral root formation is not due to a general defect related to IAA conjugate resistance.

The lateral root formation defect is not due to a complete inability to form lateral roots, since \textit{ilr2-1} does respond to lateral root promoting stimuli such as high temperature and the lateral root promoting auxin indole-3-butyric acid (IBA; Figure III-6B). Even though IBA does induce lateral roots in the mutant the number of lateral roots formed is still less than wild type. However, growth on IAA-amino acid conjugates appears to rescue of \textit{ilr2-1}'s lateral root defect. Given that all conjugates tested promote lateral root formation in the mutant but not in the wild-type, it is hard to understand the significance of this result, particularly since IAA-Ala, to which \textit{ilr2-1} is not resistant in root elongation, can also rescue the lateral root defect. However, further attempts to identify a concentration at which IAA conjugates induce lateral roots in wild-type seedlings were futile.
Figure III-6. *ilr2-1* has a low number of lateral roots, which can be rescued by IAA-conjugates. The number of lateral roots on seedlings from the indicated genotypes was counted after eight days of growth (A) at 22 °C or the auxin-inducing temperature 28 °C; or (B) at 22 °C in media supplemented with the indicated hormones. Alleles used for (A) are: *ilr2-1, iar1-1, iar3-2, ilr1-1*. Error bars represent standard errors of the means (n ≥ 10).
III.F. High temperature induces wild-type root and hypocotyl elongation in *ilr2-1*

In contrast to the defects in primary root elongation and in lateral root formation, other auxin-related phenotypes appear normal in the *ilr2-1* mutant. High temperature promotes auxin accumulation (Gray et al. 1998) and promotes lateral root formation, and primary root (Rogg et al. 2001) and hypocotyl elongation (Gray et al. 1998). The hypocotyl and root elongation phenotypes were tested in the *ilr2-1* mutant, which was found to respond like wild type (Figure III-7). In addition, other IAA-amino acid resistant mutants were analyzed at high temperatures and they also exhibit wild-type root and hypocotyl growth induction (Figure III-7).

In conclusion, *ilr2-1* exhibits fewer lateral roots than wild type, a condition that becomes more apparent when grown at high temperature, which promotes lateral roots. However, *ilr2-1* is not deficient in all high temperature responses; *ilr2-1* roots (Figure III-7A) and hypocotyls (Figure III-7B) elongate in response to high temperature. It is possible that the low number of lateral roots exhibited by *ilr2-1* reflects a lower concentration of free IAA, compared to wild-type, which is why addition of conjugates can rescue this phenotype (Figure III-6). These results further support the hypothesis that when Arabidopsis seedlings are exposed to conditions that induce IAA accumulation, the seedlings achieve this accumulation through different input pathways, that may be used differentially in a variety of cells or tissues through development, further reflecting the dynamic nature of IAA homeostasis.
Figure III-7. IAA-amino acid resistant mutants exhibit wild-type root and hypocotyl elongation responses to high-temperature auxin induction. Seedlings from wild-type Ws or the indicated IAA-amino acid resistant mutants were grown for eight days at 22 °C (black bars) or at 28 °C (hatched bars), after which their (A) primary root length or (B) hypocotyl length was measured. Error bars represent standard errors of the means (n ≥ 10).
III.G. *ilr2-1* is sensitive to 2,4-D and auxin transport inhibitors

Because lateral root formation requires auxin transport (Reed et al. 1998; Casimiro et al. 2001), the lateral root defect observed in *ilr2-1* suggested the possibility that *ilr2-1* may have auxin transport defects. IAA is the most common naturally occurring auxin, but other compounds that exhibit auxin effects and enter and exit the cells through different mechanisms than IAA have been identified. These include the synthetic auxins 2,4-D and NAA (Bennett et al. 1996). 2,4-D enters cells through AUX1, the auxin influx carrier, but does not require the PIN transporters to exit cells. Mutants with defects in the auxin transport machinery can exhibit altered sensitivity to 2,4-D or to the compounds known as auxin transport inhibitors. Therefore, I analyzed the effects of 2,4-D and auxin transport inhibitors on root growth of *ilr2-1*. The results, shown in Figure III-8, demonstrate that *ilr2-1* exhibits a wild-type response to the synthetic auxin 2,4-D and to the auxin transport inhibitors. These results suggest that the *ilr2-1* resistance to IAA-Leu, as well as its lower number of lateral roots, is not due to a defect in hormone transport.
Figure III-8. *ilr2-1* exhibits a wild-type response to the synthetic auxin 2,4-D and auxin transport inhibitors. Wild type Ws and *ilr2-1* mutant seedlings were grown for eight days in the indicated compounds, after which their primary root length was measured. Error bars represent standard errors of the means ($n \geq 8$).
III.H. *ilr2-1* is sensitive to other phytohormones

The majority of the auxin resistant mutants that have been identified are pleiotropic (see Chapter I). This pleiotropism usually includes, but is not limited to, resistance to other phytohormones. Given that the *ilr2-1* mutant is resistant to some forms of auxin, I was interested in determining whether it exhibited resistance to other phytohormones. Towards this end, I analyzed the root or hypocotyl elongation of *ilr2-1* and wild type seedlings in the presence of cytokinin, brassinosteroids, jasmonic acid, the jasmonic acid conjugate JA-Phe, and the ethylene precursor ACC. The results, shown in Figure III-9, indicate that *ilr2-1* exhibits a wild-type response to these phytohormones.

The effect of the phytohormone ABA was also analyzed. ABA inhibits germination; therefore, it was of interest to determine the effect of ABA on the root growth of *ilr2-1*, given its germination defect. Toward this end, I analyzed the root elongation of wild-type Ws and *ilr2-1* mutant seedlings upon transfer to different concentrations of ABA. The root length of the transferred seedlings was analyzed at different time intervals to obtain an estimation of the root growth rate of *ilr2-1* and wild type on ABA. The results are shown in Figure III-10. The roots of *ilr2-1* do appear to be more sensitive to ABA at any concentration analyzed (Figure III-10A-C), perhaps because *ilr2-1* has a slower growing root than wild-type. However, the sensitivity to a range of concentrations four days after transfer, as determined by the shape of the curves for wild type and *ilr2-1*, clearly indicates that *ilr2-1* is not hypersensitive to ABA (Figure III-10D). Therefore, the apparent hypersensitivity to ABA is an effect of *ilr2-1*’s slower root-growth rate.
Figure III-9. *ilr2-1* has a wild-type response to tested phytohormones. Wild-type *Ws* (black bars) or *ilr2-1* (hatched bars) seeds were plated on (A) methyl jasmonate (MeJA) or the jasmonate conjugate JA-Phe, (B) epibrassinolide (eBR), (C) cytokinin benzyl-adenine (BA), (D) ethylene precursor ACC, or (E) gibberellic acid (GA3). After eight days, the primary root length (A,B,C and D) or hypocotyl length (E) was measured. Error bars represent standard errors of the means (*n* ≥ 6).
Figure III-10. Roots of ilr2-1 exhibit a wild-type response to root growth inhibition mediated by ABA. Four-day old wild-type Ws or ilr2-1 seedlings were transferred from unsupplemented media to media containing (A) 3 μM ABA, (B) 5 μM ABA, or (C) 10 μM ABA. The plates were oriented vertically, allowing the roots to grow downward over the media surface. At the specified times, the length of the primary root was recorded. (D) Representation of compiled data for four days after transfer, on the indicated concentrations of ABA. Error bars represent the standard errors of the means (n ≥ 8, except for C, where n ≥ 4).
In conclusion, *ilr2-1* exhibits a wild-type response to all the known phytohormones, in addition to its wild-type response to the free form of IAA.

**III.I. *ilr2-1* is resistant to root inhibition by cobalt and manganese**

In addition to its well-known association with other phytohormones, auxin homeostasis, in particular IAA-conjugate metabolism, has been linked with the effect of ions (see Chapter I). Therefore, I compared the responses to growth on ions of the *ilr2-1* mutant to those of wild type. For these comparisons I chose two ions that can function as cofactors for the amidohydrolases, manganese and cobalt, and two ions that inhibit the manganese-dependent IAR3 amidohydrolase activity, zinc and copper (Lasswell 2000). The results, shown in Figure III-11, indicate that *ilr2-1* is resistant to cobalt and manganese, but responds normally to zinc and copper.

The media used to grow plants in the laboratory contains a low concentration of these ions, in addition to a variety of others. These concentrations are represented in Table III-2. Please note that the concentrations presented in Figure III-11 indicate the ion concentration added to the media, rather than the total ion concentration present in the media.
Figure III-11. The *ilr2-1* mutant is resistant to the effects of cobalt and manganese. Seeds from the wild-type (Ws) or *ilr2-1* were sown on media lacking sucrose supplemented with the indicated amounts of (A) CoCl$_2$, (B) CuSO$_4$, (C) MnCl$_2$, or (D) ZnSO$_4$. After eight days, the length of the primary root was measured. Note that the ion concentrations indicated on the horizontal axes refer to the amount of metal added to the media, not to the total metal present in the media. Error bars represent standard errors of the means ($n \geq 7$).
Table III-2. Ion concentration in unsupplemented plant growth medium.

<table>
<thead>
<tr>
<th>Ion</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cobalt</td>
<td>0.01 μM</td>
</tr>
<tr>
<td>Molybdenum</td>
<td>0.2 μM</td>
</tr>
<tr>
<td>Copper</td>
<td>0.5 μM</td>
</tr>
<tr>
<td>Sodium</td>
<td>10.4 μM</td>
</tr>
<tr>
<td>Manganese</td>
<td>14 μM</td>
</tr>
<tr>
<td>Chloride</td>
<td>48 μM</td>
</tr>
<tr>
<td>Iron</td>
<td>50 μM</td>
</tr>
<tr>
<td>Boron</td>
<td>70 μM</td>
</tr>
<tr>
<td>Zinc</td>
<td>1 μM</td>
</tr>
<tr>
<td>Calcium</td>
<td>2 mM</td>
</tr>
<tr>
<td>Magnesium</td>
<td>2 mM</td>
</tr>
<tr>
<td>Sulfate (SO₄)</td>
<td>2 mM</td>
</tr>
<tr>
<td>Phosphate (PO₄)</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>Potassium</td>
<td>7.5 mM</td>
</tr>
<tr>
<td>Nitrate (NO₃)</td>
<td>9 mM</td>
</tr>
</tbody>
</table>
Given the resistance that *ilr2-1* roots exhibit towards cobalt and manganese, I wanted to determine whether this effect was specific for *ilr2-1* or shared by other IAA-amino acid resistant mutants. Therefore, I analyzed the effect of growth on cobalt and manganese on the root elongation of the other IAA-amino acid resistant mutants *iar1, iar4,* and of the double hydrolase mutant *ilr1 iar3*.

The results shown in Figure III-12A indicate that cobalt-resistant root growth is specific for *ilr2-1*. Although *iar4*, which has a short root on unsupplemented medium, does not exhibit an inhibition equivalent to that of wild type, its root is still not as resistant to cobalt as *ilr2-1*. In other words, IAA-amino acid resistance is not generally linked to cobalt resistance; however, *ilr2-1* is resistant to both IAA-Leu and cobalt, perhaps through the same mechanism.

The results shown in Figure III-12B indicate that all IAA-amino acid resistant mutants exhibit manganese resistance, albeit to varying degrees. This suggests that at least part of the effects of manganese on seedling root growth is mediated through IAA-amino acid hydrolysis. However, no mutant is as resistant to manganese as *ilr2-1*, which further suggests that *ilr2-1* has a defect in metal homeostasis; this defect appears to relate specifically to cobalt and manganese homeostasis.

Manganese and cobalt can serve as cofactors for the amidohydrolases (LeClere et al. 2001); therefore, it seems reasonable to speculate that *ilr2-1* is resistant to the effects of conjugates because it has a metal homeostasis defect. Cobalt and manganese homeostasis appear to be linked (see section III.L), and a defect in cobalt homeostasis may lead to manganese resistance.
Figure III-12. *ilr2-1* is the only IAA-amino acid resistant mutant that is resistant to manganese and cobalt mediated root inhibition. Seeds from known IAA-amino acid resistant mutants were sown on media lacking sucrose supplemented with the indicated concentrations of (A) CoCl₂ or (B) MnCl₂. After eight days, the primary root of the seedlings was measured. The error bars represent standard errors of the means (*n* ≥ 8).
The cobalt and manganese resistance of ilr2-1 prompted us to analyze the effects of other ions on root growth. I analyzed ions that, like cobalt, are contained in the growth media. I tested iron (in the form of Fe-EDTA) and potassium. Fe-EDTA has deleterious effects on the green parts of the plant, causing bleaching and reduced cotyledon growth, whereas it has little effects on the roots at the concentrations tested which ranged from 100 nM to 7 μM Fe-EDTA. Therefore, I was not able to test whether ilr2-1 roots are resistant to the effects of Fe-EDTA. I was able to compare the effect of potassium on root elongation between wild type and ilr2-1; these results, shown in Figure III-13, show that ilr2-1 exhibits a wild-type response to potassium. Given that the concentrations tested range from 10 to 150 mM KCl, these results also show that ilr2-1 responds normally to the osmotic stress induced by KCl.

III.1. Cobalt and zinc can rescue the short-root phenotype of ilr2-1

Analyzing the effects of ions on ilr2-1 root growth, we realized that low concentrations of manganese can induce root elongation in ilr2-1 (Figure III-12B). We therefore analyzed whether low concentrations of other ions can also rescue the short-root phenotype of ilr2-1. We found that cobalt and zinc, but not iron (Figure III-14) or copper (Figure III-11B), can rescue the short-rooted phenotype of ilr2-1. This is an interesting result because ilr2-1 has different root-elongation responses to root inhibition mediated by cobalt, manganese, and zinc. It is worth noting that whereas cobalt and zinc induce elongation of ilr2-1 at concentrations that inhibit wild type and the IAA-amino acid resistant mutant iarl, Fe-EDTA induces elongation of iarl. These results further indicate the link between metal homeostasis and IAA-amino acid resistance.
**Figure III-13.** *iir2-1* does not have a defect in potassium homeostasis or osmosensitivity. Wild-type *Ws* and mutant *iir2-1* seeds were sown on media lacking sucrose with the indicated concentrations of KCl. After eight days, the primary root growth was measured. The error bars represent standard errors of the means ($n \geq 6$).
Figure III-14. The short-rooted phenotype of *ilar2-1* can be rescued by low concentrations of cobalt and zinc, but not iron. Wild-type (Ws), *ilar2-1*, and *iar1-1* seedlings were grown in the indicated concentrations of cobalt, zinc, and iron for eight days before measuring the length of the primary root. Bars represent standard errors of the means (*n* ≥ 6).
III.K. The resistance to cobalt is not related to ethylene

Among the known effects of cobalt is the inhibition of the ethylene biosynthetic pathway (Kieber et al. 1993). Cobalt inhibits ethylene biosynthesis by inhibiting ACC synthase, which catalyzes the conversion of S-adenosyl methionine (SAM) to ACC. ACC is then oxidized by ACC oxidase to form ethylene (Johnson and Ecker 1998). In addition to this link between cobalt and ethylene, it is known that auxin induces transcription of a gene encoding an ACC synthase. Given this information, it seemed possible that the ilr2-1 resistance to cobalt was related to a defect in the ethylene biosynthetic pathway.

To determine whether ilr2-1 resistance to cobalt was related to the ethylene biosynthetic pathway, we tested the effect of other inhibitors of this pathway on the root and hypocotyl growth of ilr2-1 seedlings. The inhibitors tested were aminooxyvinylglycine (AVG), which inhibits ACC synthase, and α-aminoisobutyric acid (AIB), which inhibits ACC oxidase (Kieber et al. 1993). We tested the effects of these compounds on dark-grown seedlings, because the effects of ethylene are better observed in the dark than in the light (Davies 1995). The results of this experiment are shown in Figure III-15. We also analyzed the ethylene overproducing mutant etol as a control, because it is known that etol hypocotyl growth defect can be rescued by the addition of ethylene-biosynthesis inhibitors (Roman et al. 1995). The ilr2-1 mutant is not resistant to either ethylene (Figure III-9) or the tested ethylene biosynthetic inhibitors, but paradoxically it exhibits hypersensitivity to the effects of AVG (Figure III-15). These results suggest that the cobalt resistant phenotype of ilr2-1 is not directly related to the ethylene biosynthetic pathway.
Figure III-15. Roots and hypocotyls of \( \textit{irr2-1} \) are not resistant to ethylene biosynthesis inhibitors. Wild-type (Ws), \( \textit{irr2-1} \), \( \textit{irr2-1} \) carrying a wild-type allele of \( \textit{ILR2} \), and ethylene overproducing \( \textit{eto1-1} \) mutants were plated on the indicated concentrations of the ethylene inhibitors AIB and AVG. After eight days of growth in the dark, (A) the hypocotyl or (B) the root were measured. Error bars represent standard errors of the means \( (n \geq 8) \).
The effects of AVG are not limited to ACC synthase; AVG inhibits enzymes that use pyridoxal phosphate (vitamin B6) as cofactors. This group includes, but is not limited to, enzymes that catalyze amino-group transfer reactions and other reactions that involve amino acids. It is possible that \textit{ilr2-1}'s hypersensitivity to AVG is due to accumulation of IAA-Leu, which is usually controlled in \textit{ilr2-1} through an amino transferase. In the presence of AVG, this amino transferase would be inhibited and the accumulation of IAA-Leu could be detrimental. Alternatively, it is possible that by inhibiting the Trp-amino transferase involved in the IAA biosynthetic pathway, AVG has a detrimental effect on the root growth of \textit{ilr2-1}. At the present time, it is not possible to test which one of these hypothesis is correct, but analysis of IAA-Leu accumulation in \textit{ilr2-1} in combination with a precise characterization of the enzymatic role of ILR2 may provide some answers.

\textbf{III.L. Metal content of \textit{ilr2-1}}

The cobalt and manganese resistance of \textit{ilr2-1} could be due to an inability to import these metals, or to an altered starting concentration of these ions. To analyze these possibilities, atomic absorption analyses were performed on wild-type Ws and mutant \textit{ilr2-1} seedlings that had been grown in the absence of metals or in the presence of 100 \(\mu\text{M} \ \text{CoCl}_2\), a concentration that results in 50\% inhibition of wild-type root growth. In addition, we analyzed the double hydrolase mutant \textit{ilar iar3}, which is also resistant to IAA-Leu, in order to differentiate the effects that the \textit{ilr2-1} mutation has on metal content from the effects of IAA-Leu resistance on metal content.
From the twenty-nine analyzed metals, we identified differences in eight of them. The results for these eight metals are shown in Table III-3. The \textit{ilr2-1} mutant has a slightly higher content of cobalt than wild-type or \textit{ilr1 iar3} when grown on normal growth medium, but it does not have a defect in cobalt import because the three types of seedlings accumulate equivalent amounts of cobalt when grown on 100 \mu M CoCl\(_2\). The \textit{ilr2-1} mutant also exhibits increased concentrations of manganese when grown in normal medium. In addition to increased cobalt and manganese content in the absence of exogenously supplied cobalt, \textit{ilr2-1} also has slightly higher concentrations of chromium, iron, nickel, and thallium, whereas it has slightly lower concentrations of titanium. The significance of the specific changes in metal concentration are hard to explain given our present knowledge of their effects, but they do show that the \textit{ilr2-1} mutant has altered metal homeostasis.

It is interesting to note that when plants are grown in the presence of cobalt, the concentration of other metals changes (Table III-3): cobalt induces a decrease in the concentration of manganese, chromium, and potassium, whereas it induces an increase in the concentration of iron and titanium. The relation between cobalt and manganese is of particular interest, not only because \textit{ilr2-1} is resistant to both, but also because cobalt and manganese can function as cofactors for the IAA-amino acid hydroxylases (see Chapter I). This result suggests that cobalt and manganese share cellular transporters, or mechanisms leading to regulation of these transporters, which are probably altered in the \textit{ilr2-1} mutant. After growth on exogenous cobalt, the ion content of \textit{ilr2-1} does not appear to be any different from wild type.
<table>
<thead>
<tr>
<th></th>
<th>Cobalt</th>
<th>Chromium</th>
<th>Iron</th>
<th>Potassium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ws</td>
<td>0.9 ± 0.1</td>
<td>35 ± 18</td>
<td>333 ± 93</td>
<td>60993 ± 4052</td>
</tr>
<tr>
<td><em>ir2-1</em></td>
<td>2.0 ± 0.2</td>
<td>61 ± 13</td>
<td>605 ± 75</td>
<td>49410 ± 6955</td>
</tr>
<tr>
<td><em>ir1 iar3</em></td>
<td>0.4 ± 0.1</td>
<td>8 ± 4*</td>
<td>207 ± 68</td>
<td>55793 ± 8734</td>
</tr>
<tr>
<td>Ws CoCl₂</td>
<td>948 ± 53</td>
<td>22 ± 16</td>
<td>519 ± 162</td>
<td>30970 ± 2125</td>
</tr>
<tr>
<td><em>ir2-1</em> CoCl₂</td>
<td>929 ± 48</td>
<td>20 ± 22</td>
<td>526 ± 192</td>
<td>27376 ± 2143</td>
</tr>
<tr>
<td><em>ir1 iar3</em> CoCl₂</td>
<td>824 ± 97</td>
<td>14 ± 12</td>
<td>349 ± 107</td>
<td>52173 ± 11536*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Manganese</th>
<th>Nickel</th>
<th>Titanium</th>
<th>Thallium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ws</td>
<td>236 ± 14</td>
<td>29 ± 12</td>
<td>1.9 ± 0.9</td>
<td>12 ± 5</td>
</tr>
<tr>
<td><em>ir2-1</em></td>
<td>425 ± 18*</td>
<td>54 ± 14</td>
<td>0.4 ± 0.5</td>
<td>24 ± 4</td>
</tr>
<tr>
<td><em>ir1 iar3</em></td>
<td>212 ± 24</td>
<td>10 ± 5</td>
<td>2.2 ± 0.1</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>Ws CoCl₂</td>
<td>78 ± 0.1</td>
<td>24 ± 13</td>
<td>1.5 ± 1.5</td>
<td>24 ± 3</td>
</tr>
<tr>
<td><em>ir2-1</em> CoCl₂</td>
<td>77 ± 6.1</td>
<td>21 ± 16</td>
<td>4.0 ± 5.9</td>
<td>26 ± 3</td>
</tr>
<tr>
<td><em>ir1 iar3</em> CoCl₂</td>
<td>65 ± 12.8</td>
<td>16 ± 9</td>
<td>4.7 ± 6.3</td>
<td>15 ± 4</td>
</tr>
</tbody>
</table>

Wild-type Ws and mutant *ir2-1* seedlings were grown in media with or without 100 μM CoCl₂, as indicated. After ten days the seedlings were removed from the growth media and the tissue was dried for atomic absorption analysis. The numbers represent ppm ± std. error, as calculated from three experimental repetitions. Asterisks indicate data groups for which there is a significant difference from Wt grown in the same condition (T-test p < 0.05).

**III.M. Possible roles for ILR2**

The *ir2-1* mutant is resistant to IAA-Leu, but not to IAA-Ala or IAA. It is also resistant to the effects of cobalt and manganese, but not to other ions. In addition to these effects, the mutation results in characteristic phenotypes in the mutant, even when grown in the absence of exogenous metals or hormones: a short root and fewer lateral roots.

These phenotypes probably result from the metal and IAA homeostasis defects respectively because addition of low concentrations of metals can rescue the short-root defect (Figure III-14), and addition of low concentrations of IAA conjugates can rescue the lateral root defect (Figure III-6).

Three hypotheses can explain the *ir2-1*-mediated relationship between IAA-Leu and metal homeostasis. First, ILR2 could be important for some aspect of auxin conjugate sensitivity, and affecting this aspect in the *ir2-1* mutant has indirect effects on metal homeostasis. This hypothesis assumes that part of the effects of metals on plant
growth is mediated through hormones, specifically through auxin conjugates. This possibility seems unlikely given that other IAA-Leu resistant mutants, such as the double amidohydrolase mutant ilr1 iar3, are not resistant to the inhibitory effects of cobalt. The second hypothesis is that ILR2 directly influences metal homeostasis, and its effects on conjugates are secondary. This second hypothesis seems likely given that addition of metals can rescue the short root phenotype; furthermore, the amidohydrolases that cleave IAA-amino acid conjugates use metals as cofactors: ILR1, which preferentially cleaves IAA-Leu, prefers cobalt as its cofactor, whereas other amidohydrolases, which cleave IAA-Ala, prefer manganese (S. LeClere. personal communication). Therefore, an alteration in cobalt homeostasis could result in improper function of ILR1, and through an indirect alteration in the manganese homeostasis, cobalt can affect other IAA-conjugate amidohydrolases that prefer IAA-Ala but may cleave IAA-Leu. Alternatively, a third hypothesis is that ILR2 is important for the activity that links together metal and IAA-Leu homeostasis. As discussed in the introduction, several lines of evidence suggest a tight relationship between metal homeostasis and phytohormone regulation. This relationship has not been explored in detail and its molecular components are unknown.

At the present time, it is not possible to determine which one of these hypotheses is correct, because the biochemical activity of ILR2 remains unknown. However, it seems more likely that ILR2 affects cobalt homeostasis and has an indirect effect on auxin conjugate sensitivity. Unfortunately, very little is known about the effects of cobalt in plants, but mutants such as ilr2-1, which are resistant to cobalt, may be useful in identifying the mechanisms important for cobalt tolerance and possibly lead to applications in bioremediation.
CHAPTER IV. GENETIC AND MOLECULAR

CHARACTERIZATION OF *ILR2*

Understanding the role of *ILR2* in IAA conjugation and deconjugation requires analysis of the *ILR2* gene and the protein it encodes. This chapter describes the molecular mapping and cloning of the *ILR2* gene, the identification of a polymorphism in the *ILR2* gene, and the phenotype of *ilr2-2*, a mutant in the second form of the *ILR2* gene. In addition, this chapter presents the characterization of the *ILR2* transcript and the analysis of *ILR2* expression, and concludes with a discussion of the findings.

IV.A. Molecular mapping of *ILR2*

To determine the molecular nature of the *ilr2-1* mutation, I first mapped the gene defective in the *ilr2-1* mutant in preparation for cloning. I used recombination mapping to analyze F$_2$ populations derived from crosses between the *ilr2-1* mutant (in the Ws accession) to wild-type plants of Col-0 and Ler accessions. DNA from IAA-Leu resistant F$_2$ seedlings was analyzed using previously identified PCR-based polymorphic markers (Konieczny and Ausubel 1993; Bell and Ecker 1994). Through this initial analysis, I determined that *ilr2-1* maps to the top arm of chromosome III, specifically to a region of approximately 6 Mb contained between the markers *GL1* and nga162 (Bell and Ecker 1994). Subsequently, I developed additional PCR-based markers using sequence information provided by the AGI (Kaul et al. 2000) to narrow the *ilr2-1* mapping region (Table IV-1). Through the analysis of approximately 1280 chromosomes, I was able to narrow the mapping interval to an 105 kb region with low recombination contained
between the markers MIE15 and MSH2 (Figure IV-1). Despite further attempts to
narrow this mapping interval, no additional recombinant chromosomes were identified at
any of four new markers developed between MIE15 and MSH2 (MYF markers listed in
Table IV-1).

**Table IV-1. New PCR markers developed for mapping *ILR2***

<table>
<thead>
<tr>
<th>Marker</th>
<th>Enzyme</th>
<th>Size of products (bp)</th>
<th>Oligonucleotides</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td><strong>Col-0</strong></td>
<td><strong>Ler</strong></td>
</tr>
<tr>
<td>MSL1</td>
<td>HincII</td>
<td>240, 215</td>
<td>555</td>
</tr>
<tr>
<td>K14A17</td>
<td></td>
<td>110</td>
<td>90</td>
</tr>
<tr>
<td>MEB5</td>
<td>TaqI</td>
<td>880</td>
<td>880</td>
</tr>
<tr>
<td>MIE15</td>
<td></td>
<td>900</td>
<td>900</td>
</tr>
<tr>
<td>MYF (5+6)</td>
<td>Tsp45</td>
<td>220</td>
<td>220</td>
</tr>
<tr>
<td>MYF (7+8)</td>
<td>BstBI</td>
<td>60, 60</td>
<td>120</td>
</tr>
<tr>
<td>MYF (9+10)</td>
<td>XcmI</td>
<td>235</td>
<td>240</td>
</tr>
<tr>
<td>MYF (11+12)</td>
<td>Rsal</td>
<td>1000</td>
<td>400, 600</td>
</tr>
<tr>
<td>MSH2</td>
<td></td>
<td>500</td>
<td>350, 150</td>
</tr>
<tr>
<td>MVE11</td>
<td>Tsp509</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>MXL8</td>
<td>Ddef</td>
<td>260</td>
<td>260</td>
</tr>
</tbody>
</table>
Figure IV-1. Positional cloning of *ilr2-1*. The *ilr2-1* mutation was localized to chromosome III using the PCR-based markers nga162 (Bell and Ecker 1994) and *GL1* (Konieczny and Ausubel 1993). To further refine this position, additional PCR-based markers were developed, and *ILR2* was mapped between the markers MIE15 and MSH2, which are contained in the BACS F23E22 and T10F23. A complementation library was constructed from the BAC T10F23, and the subclones were tested for *ilr2-1* IAA-Leu resistance phenotypic rescue. The clones that did not rescue the mutant phenotype are indicated as gray boxes. Clones pBT10-2 and pBT10-132, indicated as black boxes, rescued the *ilr2-1* mutant phenotype. Of the 21 predicted ORFs contained in the MIE15-MSH2 interval (thin arrows), only one was contained in both clones pBT10-2 and pBT10-132. Upon sequence examination of the *ilr2-1* mutant, a C to T mutation (asterisk) resulting in a Pro to Leu change was identified in this gene (*ILR2*).
IV.B. Cloning of the gene defective in *ilr2-1*

The 105 Kb region to which *ilr2-1* maps is contained within the P1 clones MYF24 and MIE15. These clones were sequenced as part of the AGI (Salanoubat et al. 2000), but the sequence was not annotated. To manually annotate this sequence, I used Genscan and Grai software (Burge and Karlin 1997; Hyatt et al. 2000). Through combining the results of these two programs, I defined 21 putative ORFs within this interval. The P1 clones MYF24 and MIE15 were not publicly available, but the *ilr2-1*-mapping region was spanned by two publicly available BACs, T10F24 and F23E22 (Kaul et al. 2000). Because 18 of the 21 putative genes were completely contained within BAC T10F24, I selected this BAC to construct a complementation library.

The complementation library consisted of 300 clones that were analyzed using a combination of restriction digests and colony hybridizations (see Chapter II). This approach allowed me to select a set of 21 single clones, which together contained 17 of the 18 predicted ORFs from T10F24 (Figure IV-1). I transformed each of the 21 clones into the *ilr2-1* mutant using Agrobacterium-mediated transformation. I sequenced the only predicted ORF not contained in the complementation library along with two of the three genes not contained in T10F24 from the *ilr2-1* mutant, and found no mutations in any of these three ORFs.

*ilr2-1* T$_2$ seedlings transformed with individual plasmids from the library were tested for IAA-Leu sensitivity to assay for phenotypic restoration. I identified two constructs that completely rescued the *ilr2-1* mutant phenotype: constructs pBT10-132 and pBT10-2. The only apparent ORF shared by these two constructs had different exons
intron structures as predicted by the two software programs used (Figure IV-2). Genscan predicted an ORF consisting of three exons, the first two of which were shared by the Grail predicted four-exon ORF. Only the first two exons, shared by both predictions, were contained in the pBT10-132 rescuing construct. I sequenced this putative gene from the ilr2-I mutant and identified a C to T mutation, consistent with EMS mutagenesis, which changes a Pro to a Leu residue in the predicted second exon. Thus, this gene represents ILR2, a novel gene without significant homology to previously sequenced genes. Construct pBT10-132 is less effective at rescuing ilr2 IAA-Leu resistance than construct pBT10-2, indicating that the 3' end of the gene, absent in pBT10-132, may be of importance for ILR2 function.

The rescuing construct pBT10-2 not only rescues the IAA-Leu resistance phenotype, but also rescues the cobalt resistance (Figure IV-3) and the AVG hypersensitivity (Figure III-15). These results further confirm that this is the gene defective in the ilr2-I mutant, and that the IAA-Leu resistance, cobalt resistance, and AVG hypersensitivity are caused by a mutation in ILR2.
**Figure IV-2. ILR2 gene structure.** The rescuing constructs pBT10-132 and pBT10-2 (open rectangles) share only one ORF (filled arrows), in which a C to T mutation was identified in the ilr2-1 mutant (white asterisk). The Grail and Genscan software programs predict different exon (arrows) intron (thin lines) structures for this ORF. Both of these predictions are different from the RT-PCR and 5′RACE sequence obtained from wild-type Ws, in which a 5′ deletion was identified in the genomic DNA (see text).
Figure IV-3. The constructs pBT10-2 and pBT10-132 rescue the \textit{ilr2-1} mutant phenotypes. (A) Genomic clones pBT10-2 and pBT10-132 containing the wild-type (Col-0) \textit{ILR2} rescue the \textit{IAA-Leu} resistance of \textit{ilr2-1}. Homozygous lines of \textit{ilr2-1} transformed with clones pBT10-2 and pBT10-132 were grown on media with or without IAA-Leu. After eight days, the seedlings were taken out of the media and the length of the primary root compared to that of wild type (Ws) and \textit{ilr2-1} mutant seedlings. (B) Genomic clone pBT10-2 rescues the cobalt resistance phenotype of \textit{ilr2-1}. Homozygous lines of \textit{ilr2-1} transformed with pBT10-2 were grown on media lacking sucrose with or without CoCl$_2$. Root growth of eight-day-old seedlings was compared to that of wild type (Ws) and \textit{ilr2-1} mutant seedlings. For (A) and (B) error bars represent standard errors of the means ($n \geq 8$).
No homologs to the full or partial sequence of ILR2 could be found in the databases. Furthermore, ILR2 does not appear to have any transmembrane domains or subcellular targeting signals, according to available software prediction programs. Using a variety of sequence analysis programs available on the web, only Pfam (Bateman et al. 2000) detected an ARID (A/T Rich Interacting Domain) DNA-binding domain in the amino terminus of ILR2 (Figure IV-4). Members of the ARID protein family exhibit both specific and non-specific DNA binding activity (Gregory et al. 1996; Dallas et al. 2000), and have been implicated in functions ranging from cell lineage gene regulation to chromatin remodeling (Kortschak et al. 2000). No reports of plant ARID proteins are present in the literature. The ARID domain is poorly defined. only five amino acids are strictly conserved among species; therefore, it is difficult to predict whether the ILR2 ARID homology represents a true DNA-binding domain.
Figure IV-4. ILR2 is similar to ARID DNA-binding proteins. Protein alignment of ILR2 with five representative members of the ARID family. Yeast (Sc) ADR6, Drosophila (Dm) DEADRINGER, murine (Mm) jumonji, and human (Hs) SWI/SNF-related p270 were all identified based on their ability to bind DNA in vitro (Kortschak et al. 2000). Ce T23D8.8 is an uncharacterized C. elegans gene product. Proteins were aligned with the MegAlign program (DNASTAR). The five strictly conserved residues for the ARID family are boxed in black, and similar amino acids are shaded with gray.
IV.C. \textit{ILR2} is polymorphic between Arabidopsis ecotypes

Further analysis of the \textit{ILR2} gene in the \textit{ilr2-1} mutant revealed that the predicted first exon was deleted in the mutant and in the parental wild-type Ws, indicating that this deletion is not part of the \textit{ilr2-1} mutation. Sequencing \textit{ILR2} from wild-type Ws and \textit{ilr2-1} revealed that both genotypes contain a version of \textit{ILR2} with a 983-bp deletion in the 5' end of the Col-0 gene (Figure IV-2 and Figure IV-5). Thus, there are two forms of \textit{ILR2}, the long form (long \textit{ILR2}, from Col-0) and the short form (short \textit{ILR2}, from Ws). It is interesting to note that the 983 bp missing in the short \textit{ILR2} encode the predicted ARID DNA-binding domain (Figure IV-5C). However, both forms of the gene are functional, because mutations in the short form cause the \textit{ilr2-1} mutant phenotype in the Ws background, and this phenotype can be rescued by the long \textit{ILR2} from Col-0, which is contained in the rescuing constructs pBT10-2 and pBT10-132.

In an effort to determine which \textit{ILR2} form is most prevalent in nature, I analyzed the \textit{ILR2} locus in 12 additional Arabidopsis accessions representative of different clades (Table II-1; Miyashita et al. 1999). PCR amplification of an \textit{ILR2} fragment that is 1139 bp in long \textit{ILR2} (Col-0) and 156 bp in short \textit{ILR2} (Ws) indicated that most ecotypes contain the long form of \textit{ILR2}, except for Ws and Ler (Figure IV-5), suggesting that the long \textit{ILR2} is the ancestral form of the gene.
Figure IV-5. *ILR2* is polymorphic between Arabidopsis ecotypes. (A) Schematic of the two forms of *ILR2* found in different Arabidopsis ecotypes. Predicted *ILR2* exons are indicated by thick arrows, and the primers used for PCR amplification in panel B are indicated by small arrows. The region represented by the hatched box is deleted in theWs and Ler ecotypes. (B) PCR analysis of the *ILR2* locus in 14 Arabidopsis ecotypes. Products resulting from PCR amplification of the long *ILR2* (1120 bp) or the short *ILR2* (137 bp) using primers shown in panel A were electrophoresed on an 1% agarose gel and photographed. (C) Predicted Col-0 *ILR2* sequence. The predicted Col-0 ILR2 peptide includes the predicted short ILR2 (represented in gray) and an ARID DNA binding domain (underlined); the five strictly conserved amino acids from this domain are in bold. The Pro mutated in *ilr2-1* to a Leu is boxed.
To determine if this polymorphism is correlated with the IAA-Leu sensitivity of wild-type seedlings, I analyzed the IAA-Leu induced root growth inhibition in ecotypes with different forms of *ILR2*. The results, shown in Figure IV-6, indicate that there is not a strict correlation between the *ILR2* form and the IAA-Leu sensitivity because both *Ler* and *Ws* contain short forms of *ILR2*, but only *Ws* exhibits an IAA-Leu sensitivity that is significantly different from that of Col-0. This result further indicates that both forms of the *ILR2* gene are functional; furthermore, it suggests that any difference in IAA-Leu sensitivity observed between *Ws* and Col-0 is probably due to a combination of factors that may or may not include *ILR2*.

Different Arabidopsis accessions have different cobalt sensitivity. Figure IV-7 shows the average root elongation of wild-type Col-0, wild-type *Ws*, and mutant *ilr2-1* seedlings grown over a gradient of cobalt concentrations. Col-0 seedlings appear more resistant to cobalt than *Ws* seedlings do, but *ilr2-1* is more resistant than either one of them.

The AGI sequenced the Col-0 accession DNA, providing a powerful tool for Arabidopsis and plant research (Kaul et al. 2000). Differences in the DNA from various Arabidopsis accessions have proven to be a useful tool in analyzing and understanding differences in the response to environmental signals, such as light (Maloof et al. 2000). The identified polymorphism in *ILR2* indicates that differences found between the Arabidopsis ecotypes may also be useful for identifying important regions of pioneer proteins.
Figure IV-6. The *ILR2* polymorphism does not affect IAA-Leu sensitivity in wild-type seedlings. Wild-type Col-0, Ler, or Ws seedlings were grown on media unsupplemented or supplemented with 20 µM IAA-Leu to analyze if the form of *ILR2* is correlated with IAA-Leu sensitivity. The asterisk indicates the only significant difference identified, as compared to Col-0 root length in the equivalent conditions (Student’s T-test, p<0.005). Bars represent standard errors of the means from eight-day-old primary root length (n=12).
Figure IV-7. Wild-type Col-0 seedlings are more resistant to the effects of cobalt than wild-type Ws seedlings. Wild-type Col-0, Ws, or ilr2-1 seeds were grown on media supplemented with the indicated concentrations of CoCl2. Bars represent standard errors of the means from eight-day-old primary root length (n=9).
IV.D. A Col-0 ilr2 mutant allele is hypersensitive to cobalt

Because of the exceptional polymorphism identified for ILR2, I was interested in analyzing the effect of a mutation in the long form of ILR2. I identified a T-DNA insertional allele in a collection of mutagenized Col-0 plants made available by the Torrey Mesa Research Institute (San Diego, CA). This T-DNA insertion resides 140 bp upstream from the predicted first ATG for the long ILR2. I analyzed the effect of this mutation, ilr2-2, on IAA-Leu and cobalt sensitivity. The results shown in Figure IV-8A indicate that the ilr2-2 mutant has no effects on IAA-Leu sensitivity. However, as shown in Figure IV-8B, ilr2-2 is supersensitive to the effects of cobalt on primary root elongation. Because of the position of the T-DNA in the ilr2-2 allele, it is reasonable to assume ilr2-2 represents a loss-of-function mutation. Therefore, the ilr2-1 mutation, which has the opposite phenotypic response to cobalt from ilr2-2, may be a gain-of-function mutation.
Figure IV-8. *ilr2-2* mutant seedlings are supersensitive to cobalt and have a wild-type response to IAA-Leu. Wild-type Col-0 and different lines of *ilr2-2* seedlings were grown for eight days on media supplemented with the indicated concentrations of (A) CoCl₂ or (B) IAA-Leu. Bars represent standard errors of the means (n ≥ 8).
**IV.E. Molecular structure of the *ILR2* genes**

As mentioned earlier, the two software programs used to predict ORFs in the *ilr2-1* mapping region predicted two different exon-intron structures for Col-0 *ILR2* (Figure IV-2). To determine which of the protein predictions for ILR2 was correct, as well as the difference in the ILR2 protein coded by the Ws and the Col-0 genome, I wanted to analyze *ILR2* cDNAs. However, I was unable to isolate an *ILR2* cDNA from two different cDNA libraries made from Col-0 or Ler RNA (Minet et al. 1992; LeClere and Bartel 2001). Therefore, I performed RT-PCR and RACE PCR on Col-0 and Ws RNA to define the coding region of *ILR2*. I was unable to identify any products from Col-0, probably due to low expression of *ILR2* (See section IV.F). RT-PCR on Ws RNA amplified a product that included the predicted second intron, indicating that it is not spliced, resulting in a stop codon right after the Genscan predicted splice site (Figure IV-2). Furthermore, this predicted third exon is not required for rescue of *ilr2-1* phenotypes. In addition, this sequence is expressed on the other strand as part of the mRNAs encoding an apparent CASP-like protein (GenBank accession AY056225) immediately downstream of *ILR2* (open arrow in Figure IV-2). In conclusion, it is unlikely that this predicted third exon is part of *ILR2*.

5'-RACE PCR on Ws RNA identified the transcription start site immediately after the Ws deletion. The first ATG present in this transcript codes for a methionine in the same reading frame as the predicted Col-0 second exon (Figure IV-5C). Namely, the long *ILR2* appears to code for a protein of 396 amino acids, only the last 147 of which are present in the predicted short ILR2.
**IV.F. Expression of *ILR2***

The expression pattern of a given gene can sometimes shed light on its function. To determine the tissue distribution of *ILR2* expression, I performed RNA gel blot analysis on RNA extracted from root and shoot tissue from 10-day-old Ws seedlings and from rosette leaves, stems, siliques, and dry seeds from mature Ws plants. I identified *ILR2* transcript in aerial parts: seedling shoots and mature plant leaves and stems (Figure IV-9). I did not detect *ILR2* mRNA in roots despite the root phenotypes of the *ilr2-1* mutant and the recent report that IAA-Leu accumulates in root tissues of Arabidopsis (Kowalczyk and Sandberg 2001).

Because the *ilr2-1* mutant is resistant to IAA-Leu, I analyzed expression of *ILR2* in Col-0, Ws, and *ilr2-1* seedlings grown in the absence or presence of two concentrations of IAA-Leu (Figure IV-10). I did not detect induction or repression of *ILR2* by IAA-Leu in either Ws or *ilr2-1* seedlings. I detected similar levels of *ILR2* transcript in Ws and the *ilr2-1* mutant; however, no transcript was detected from the Col-0 *ILR2*, probably due to low expression. This low *ILR2* expression from the Col-0 ecotype is consistent with the inability to identify a Col-0 transcript either by RT-PCR or from the cDNA library. However, it is reasonable to expect that the Col-0 *ILR2* gene is transcribed, given that it can rescue the *ilr2-1* mutant.
Figure IV-9. *ILR2* is expressed in seedling shoots and mature-plant leaves. Total RNA (10 µg) isolated from roots and shoots from 10-day-old Ws seedlings; leaves, siliques, and stems isolated from adult plants; and dry seeds, was separated on a denaturing gel and transferred to a nylon membrane, and hybridized with a $^{32}$P labeled *ILR2* DNA probe. Ribosomal RNA is shown in the bottom panel as a loading control.

Figure IV-10. *ILR2* expression is undetectable in Col-0 and not induced by IAA-Leu in Ws or *ilr2-1*. Total RNA (6 µg) isolated from 12-day-old Col, Ws and *ilr2-1* seedlings grown in the absence of hormone, 4 µM or 40 µM IAA-Leu, was separated on a denaturing gel, transferred to a nylon membrane, and hybridized with a $^{32}$P labeled *ILR2* DNA probe. The bottom panel shows *UBQ10* hybridization of the same nylon membrane as a loading control.
Because the *ilr2-1* mutant is also resistant to some ions I analyzed *ILR2* induction by metals. I analyzed *ILR2* expression of wild-type seedlings exposed to metal concentrations twice as high as those that induce the maximum inhibition of root elongation in seedlings. *ILR2* expression in these conditions was compared to basal expression levels observed in the absence of supplied metals (Figure IV-11). Cobalt, copper, manganese, and zinc were analyzed because their effects on *ilr2-1* had been extensively characterized (see Chapter III). Iron was also analyzed because it has a strong effect on the aerial parts of the seedling where *ILR2* is expressed. The results, shown in Figure IV-11, indicate that *ILR2* expression may be downregulated in response to cobalt and possibly manganese. Interestingly, these are the two metals to which the *ilr2-1* mutant is resistant.

![Figure IV-11. *ILR2* expression in Ws seedlings exposed to high concentrations of metals. Total RNA (6 μg) was isolated from roots and shoots from 8-day-old Ws seedlings exposed for 17 hours to 1 mM CoCl₂, 0.1 mM CuSO₄, 1 mM Fe-EDTA, 5 mM MnCl₂, or 1 mM ZnSO₄, separated on a denaturing gel, transferred to a nylon membrane, and hybridized with a ³²P labeled *ILR2* DNA probe. Ribosomal RNA is shown in the bottom panel as a loading control.]
IV.G. Discussion

Cloning the gene defective in a mutant usually provides a variety of clues about the function of the affected gene. However, to do this, the identified gene must be similar to something previously found or be amenable to biochemical studies. I cloned the gene defective in the ilr2-1 mutant; however, this gene is a pioneer gene, with no homology to previously identified genes or ESTs in the databases. Hence, it is difficult to predict the function of ILR2 based on the gene identity.

The ILR2 gene is polymorphic between Arabidopsis ecotypes. Analysis of 14 ecotypes revealed two contain an ILR2 isoform that encodes a predicted 147 amino acid protein (Ws and Ler), whereas the other 12 contain an isoform that encodes a predicted 396 amino acid protein. My observation that a mutation in the short ILR2 can be rescued by the long ILR2 indicates that both forms of the gene are functional. The short form might be less efficient than the long form, explaining the higher accumulation of ILR2 mRNA in Ws compared to Col-0 and perhaps the higher cobalt-sensitivity of Ws compared to Col-0.

The 249 amino acids deleted from the Ws and Ler predicted proteins include the predicted ARID DNA-binding domain identified in Col-0 ILR2. There is one other example of proteins losing their ARID domain. Members of the jumonji protein family, a family of transcription factors related to the ARID protein jumonji, are found in organisms ranging from yeast to human. The majority of these members share homology outside of the DNA-binding domain, which is not conserved throughout the family. In fact, evidence suggests that the DNA-binding domain has been swapped through evolution and has been replaced for other DNA binding domains (Balciunas and Ronne
2000). It seems reasonable to speculate that, as these jumonji proteins did during the process of swapping domains, ILR2 may function in the absence of the ARID domain. It is possible that the Ws and Ler forms of ILR2 are in the process of swapping their DNA binding domains and meanwhile function by interacting with other proteins. This hypothesis would also account for the difference in mRNA expression observed between the Ws ecotype containing the short form of ILR2 and the Col-0 ecotype containing the long form of ILR2. If the short ILR2 must interact with another protein in order to function, it is reasonable to expect that it should be expressed at higher levels to favor an interaction.

Alternatively, it is possible that ILR2 is not a DNA-binding protein. The ARID domain is relatively poorly conserved with only 5 amino acids defined as strictly necessary: these amino acids are scattered within an ~100 amino acid region. This relaxed domain definition makes it difficult to predict if the conserved amino acids found in the ILR2 protein are indeed part of a DNA binding domain, or if they are serendipitously arranged in a manner similar to that observed in ARID domains. Analysis of the ILR2 protein could prove helpful in analyzing its function; however, I was unable to express this protein in E. coli using a glutathione synthase transferase fusion system. It would be of interest to attempt ILR2 protein purification using a different system in the future.

The ilr2-1 mutation, which results a Pro to Leu change, leads to cobalt, manganese, and IAA-Leu resistance. In contrast, the ilr2-2 mutation, which results from a T-DNA insertion 140 bp upstream from the first predicted ATG, results in cobalt supersensitivity: the lr2-2 mutation has no effect on the response to IAA-Leu. Because of the position of
the T-DNA insertion in *ilr2-2*, it is likely that *ilr2-2* represents a loss-of-function allele.

The *ilr2-1* mutation, with the opposite phenotypes, may represent a gain-of-function mutation. The *ilr2-1* mutation is recessive, and I was able to clone the gene by complementation, indicating that if the *ilr2-1* mutation causes a gain of function, the effects of this mutation can be rescued if enough copies of the wild-type *ILR2* are present.

Accumulation of the *ILR2* mRNA is not induced by IAA-Leu, suggesting that if *ILR2* acts as a transcription factor, the expression of the regulated genes is probably altered even without exposure to exogenous IAA-Leu. However, cobalt and probably manganese, may result in a decrease in the levels of the *ILR2* transcript. The enhanced accumulation of cobalt in the *ilr2-1* mutant, or the *ilr2-1* mutation itself, may lead to abnormal expression in response to these metals. Alternatively, *ilr2-1* may have constitutive defects in gene expression that lead to metabolism changes. These changes can affect basal functions in *ilr2-1*, explaining the combination of phenotypes identified in the mutant: its slower primary root growth, low number of lateral roots, IAA-Leu resistance, and cobalt and manganese resistance. If *ILR2* is indeed a transcription factor, DNA chip analysis should allow the identification of the transcripts whose levels are altered in the *ilr2-1* mutant. Given the cobalt resistance phenotype of *ilr2-1* and the lack of knowledge about cobalt effects in plants, it would be of interest to perform DNA chip analysis comparing *ilr2-1*, *ilr2-2*, and wild type with and without cobalt. The results of this experiment may allow the identification of genes important for cobalt homeostasis and may shed some light on the mechanisms that link auxin and metal homeostasis.
CHAPTER V. AUXIN SUPERSENSITIVE MUTANTS

Among the several mechanisms that act to maintain plant auxin homeostasis, negative regulators are presumed to exist. However, very little is known about them, and few have been characterized. To study these negative regulators, I developed a genetic screen designed specifically to identify mutants hypersensitive to IAA. Through this screen three novel auxin supersensitive (aux) mutants were identified. The screen and the mutants are discussed in this chapter.

V.A. aux genetic screen design.

Several genetic screens have identified mutants resistant to auxin, and these mutants have led to the identification of genes important for auxin homeostasis (see Chapter I). However, despite the numerous indications that negative regulators are also important for auxin homeostasis, very few of these have been identified. We reasoned that mutations in genes coding for these negative regulators would result in an auxin supersensitive phenotype.

As a first step towards studying these IAA supersensitive mutants, I designed a genetic screen that allows their identification, based on a modification of the root bending assay first described by Howden and Cobbett (1992). The screen is depicted in Figure V-1A. In this protocol, M$_2$ seeds are plated on permeable cellophane membranes placed on top of growth media. The plates are placed on a vertical position to allow the roots to grow downward over the media surface. After four or five days, the cellophane
membranes with the seedlings grown over them are transferred to new plates and rotated 180° from the initial growth direction, still in a vertical position. The plates to which the cellophane membranes with the seedlings are transferred contain growth media supplemented with concentrations of IAA that result in a 50% inhibition of wild-type-seedling root growth. In response to the change in the gravitropic vector, the roots turn 180° and continue growing. IAA supersensitivity can then be recognized by enhanced inhibition of growth or enhanced lateral root formation during the second phase of growth that occurs in the presence of IAA. Figure V-1 shows an example of a plate used for the mutant screen and a representation showing different IAA supersensitive phenotypes that I was able to detect with this screen.

**V.B. axs genetic screen results.**

Using the described screen to identify axs mutants, I screened 15,030 M₂ seedlings. From this primary screen, 459 putative mutants were identified. To eliminate mutants whose root growth defects were not due to the effects of IAA, I conducted a secondary screen through which I discarded some of these 459 mutants (Figure V-2). At least ten M₁ seeds from the 171 fertile lines identified in the primary screen were plated on growth media in the presence and absence of exogenous IAA. Root lengths were measured and compared to those of wild-type seedlings. Following this screen, 62 mutants were retained for further analysis.
Figure V-1. Mutant screen for auxin supersensitive (axs) mutants. (A) Schematic of mutant screen. Mutagenized M2 seeds were plated on top of cellophane membranes in growth media and placed vertically to allow their roots to extend downward over the surface of the plate. After four days, the membrane was transferred to media supplemented with low concentrations of IAA that result in a 50% inhibition of root-growth for wild-type seedlings. The transferred roots respond to the change in the gravitropic vector by turning 180°, thus making evident the difference in growth that occurred in the absence of IAA from growth that occurred in the presence of IAA. In this representation, seedlings 3 and 4 are IAA supersensitive mutants that exhibit either no root growth after transfer (seedling 3) or enhanced lateral root formation (seedling 4). Seedlings 1 and 2 would be discarded because of their wild type phenotype (seedling 1) or a defect not associated with IAA (seedling 2). (B) Example of mutant screen plate. Seedlings were grown for four days in hormone-free media prior to their transfer to IAA-supplemented media. Arrows point to the root hook indicative of growth after transfer. The seedling on the far left lacks this hook and is a putative axs mutant.
From the 62 mutants retained from the secondary screen, I profiled the 54 lines that were fertile enough to be analyzed using two tests: IAA-mediated inhibition of root elongation and IAA-mediated induction of secondary root formation (Figure V-2). For the first test, M3 or M4 seeds were plated on growth media supplemented with IAA concentrations ranging from 5 to 100 nM. After eight days, roots were measured and compared to the root elongation of wild-type seedlings. For the second test, wild-type and mutant seeds were initially plated on media without hormone, and then seedlings were transferred to media supplemented with different concentrations of auxin (IAA and/or IBA). After four days of growth in the presence of auxin, the number of secondary roots on each seedling was counted. This last test allowed the analysis of a positive response to auxin, namely formation of secondary roots. The results from the primary, secondary, and tertiary screens are represented in Figure V-2.

Based on the profiles obtained from the tertiary screen, I selected 14 axs mutants for further analysis (Table V-1). These lines were analyzed using additional tests: effect of high temperature on root and hypocotyl elongation and on lateral root formation, resistance to other phytohormones (cytokinin, ethylene, giberellic acid, and jasmonic acid), and effects of auxin transport inhibitors. All analyzed mutants exhibited wild-type responses to the non-auxin phytohormones studied. Other results from these tests that were unique to some analyzed mutants are presented in Table V-1. The 14 mutants were then back-crossed to the parental (Col-0) ecotype, and out-crossed to one or two other ecotypes (Ler or Ws) for mapping purposes.
Figure V-2. Outcome of primary, secondary and tertiary *axr* mutant screens. The series on the left details the number of individuals screened at each stage and the outcome of that screen. The series on the right illustrates the genetic screen sequence, with figures (primary screen) or examples (secondary and tertiary) from results obtained from these screens.
<table>
<thead>
<tr>
<th>Mutant</th>
<th>Profile data</th>
<th>Additional characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>X96</td>
<td>Auxin supersensitive in root elongation &amp; in secondary roots</td>
<td>IAA supersensitivity in transfer test</td>
</tr>
<tr>
<td>X174</td>
<td>Auxin supersensitive in root elongation &amp; in secondary roots</td>
<td></td>
</tr>
<tr>
<td>X185</td>
<td>WT root elongation, high number of secondary roots</td>
<td>High number of secondary roots (without exogenous auxin)</td>
</tr>
<tr>
<td>X207</td>
<td>Auxin supersensitive in root elongation &amp; in secondary roots</td>
<td>IAA supersensitivity in transfer test</td>
</tr>
<tr>
<td>X233</td>
<td>Auxin supersensitive in root elongation &amp; in secondary roots</td>
<td>IAA supersensitivity in transfer test</td>
</tr>
<tr>
<td>X244</td>
<td>Auxin supersensitive in root elongation &amp; in secondary roots</td>
<td>High number of secondary roots induced by high temperature</td>
</tr>
<tr>
<td>X250</td>
<td>Auxin supersensitive in root elongation &amp; in secondary roots</td>
<td>High number of secondary roots induced by high temperature</td>
</tr>
<tr>
<td>X251 (axs1)</td>
<td>Auxin resistant in root elongation, supersensitive in secondary roots</td>
<td>High number of secondary roots (without exogenous auxin); root-curling phenotype; abnormal response to ATIs.</td>
</tr>
<tr>
<td>X255 (axs2, axs3)</td>
<td>Auxin supersensitive in root elongation &amp; in secondary roots</td>
<td>High number of secondary roots induced by high temperature; root-curling phenotype.</td>
</tr>
<tr>
<td>X279</td>
<td>Auxin supersensitive in root elongation &amp; in secondary roots</td>
<td>High number of secondary roots induced by high temperature</td>
</tr>
<tr>
<td>X343</td>
<td>WT root elongation, high number of secondary roots</td>
<td>High number of secondary roots induced by high temperature</td>
</tr>
<tr>
<td>X344</td>
<td>WT root elongation, high number of secondary roots</td>
<td>High number of secondary roots (without exogenous auxin)</td>
</tr>
<tr>
<td>X355</td>
<td>WT root elongation, high number of secondary roots</td>
<td>High number of secondary roots (without exogenous auxin)</td>
</tr>
<tr>
<td>X411</td>
<td>Resistant in root elongation, supersensitive in secondary roots</td>
<td>High number of secondary roots induced by high temperature</td>
</tr>
</tbody>
</table>
After back-crossing and/or out-crossing the 14 selected \textit{axs} mutants, the F\textsubscript{2} progeny of these crosses were analyzed for each of the parental phenotypes (listed in Table V-1). Two of the mutants, X251 and X255, exhibited segregation in the out-crossed F\textsubscript{2} population for one or more of the phenotypes that had been observed in the mutagenized lines. Other crossed F\textsubscript{2} populations also segregated a supersensitive phenotype in root elongation or secondary root formation, but the difference between mutant and wild type was smaller than the difference between ecotypes; therefore, mapping these mutations would not be possible. Finally, another group of F\textsubscript{2} populations did not exhibit segregation of the phenotypes, suggesting that the phenotype resulted from two or more mutations.

\section*{V.C. \textit{axs1}, \textit{axs2}, and \textit{axs3} phenotypes}

The two \textit{axs} mutants that were identified as a result of the genetic screen, \textit{axs1} (X251) and \textit{axs2} (X255), were initially isolated in the primary screen because of their IAA supersensitivity in secondary root formation. Further analysis of \textit{axs2} revealed that this line was segregating two separate phenotypes, both interesting: the \textit{axs2} mutation, which results in an increase in lateral root formation, and the \textit{axs3} mutation, which results in high-temperature and IBA supersensitivity in root elongation. These three mutants will be discussed in detail in the remainder of this chapter.

\section*{V.C.1. \textit{axs1} exhibits abnormal root curling and root waving}

The \textit{axs1} mutant represents a puzzling case because some of the phenotypes of the original line segregate in an outcross to the Ws ecotype; however, I have not been able to identify back-crossed F\textsubscript{2} individuals exhibiting any of the \textit{axs1} phenotypes. These
phenotypes are defective root curling and root waving, and abnormal response to auxin transport inhibitors.

Despite the fact that \( axs/ \) was isolated from the screen because of increased lateral root formation, this phenotype has not been recapitulated in subsequent generations derived from this line. The \( axs/ \) mutant exhibits a wild-type response to IAA-mediated inhibition of root growth; although at very low concentrations of IAA, its primary root growth defect appears rescued (Figure V-3). \( axs/ \) exhibits other interesting root behaviors that are reminiscent of auxin effects. When Arabidopsis seedlings are grown on plant nutrient media with agar, the root of wild type seedlings grows first to the bottom of the plate, and then it starts growing in a circular pattern. This “root-curling” behavior is presumed to be a result of gravitropism, for which auxins are known to be important, and thigmotropism, the response to mechanical stimuli. Root curling is absent or abnormal in \( axs/ \) and \( axs2 \) seedlings. Another process that is related to gravitropic and thigmotropic stimuli is “root waving”. Root waving occurs when Arabidopsis seedlings are grown at a 30° angle on plant nutrient medium with hard agar, which is a medium that the roots cannot penetrate because of its firmness. When wild-type seedlings are grown on hard agar, their roots grow in a wavy pattern; \( axs/ \) and \( axs2 \) mutants grown on hard agar exhibit relaxed or absent root waving (Figure V-4).
Figure V-3. Low concentrations of IAA can rescue the short root of *axs1*. Wild-type Col-0 and *axs1* M₂ seedlings were grown in media supplemented with the indicated concentrations of IAA. After eight days, the primary root length was measured. Error bars represent standard errors from the primary root length mean (n ≥ 5).

Figure V-4. *axs1* and *axs2* have root-waving defects. Arabidopsis seedlings grown on hard agar at a 30° angle exhibit a root-waving phenotype (shown for Col-0 wild type) resulting from thigmotropic and gravitropic stimuli. The mutants *axs2* (back-crossed seedlings) and *axs1* (M₂ seedlings) exhibit a relaxed or absent root-waving pattern.
Because auxin transport is important for the gravitropic response in roots, we tested $axs/l$ response to auxin transport inhibitors. The results, shown in Figure V-5, indicate that $axs/l$ is resistant to low concentrations of the inhibitors, but not to high concentrations. This result combined with the rescue of $axs/l$ short root by low concentrations of IAA suggests that $axs/l$ may have a defect in auxin homeostasis. However, this defect is only apparent when seedlings are treated with low concentrations of IAA or of auxin transport inhibitors, probably because when the hormone or the transport inhibitors are present at high concentrations, other mechanisms can go into effect to maintain auxin homeostasis.

V.C.2. $axs2$ is hypersensitive to auxin-mediated induction of lateral roots

The second mutant isolated from the $axs$ screen, $axs2$, exhibits root-curling and root-waving defects like $axs/l$ (Figure V-4). To analyze if the mutant has an auxin transport defect, we analyzed its root growth in the presence of the auxin transport inhibitor NPA. We also analyzed its root growth in the presence of 2,4-D, an auxin that does not use the PIN-like carriers to exit the cells (Delbarre et al. 1994). The results, shown in Figure V-6 and Figure V-7, indicate that the mutant is unlikely to have an auxin transport defect because its root-growth response to both of these compounds is the same as wild type. However, $axs2$ does appear to have a faster-growing root than wild type (Figure V-6, Figure V-7), which may be the reason for the defective root-curling and root-waving phenotypes.
Figure V-5. *axs1* is resistant only to low concentrations of auxin transport inhibitors. *axs1* mutant and Col-0 wild type seedlings were grown on the indicated media for eight days and their primary root measured. Error bars represent standard deviations of percentage root elongation compared to unsupplemented medium (*n* ≥ 10). Data supplied by Andrew Woodward.
Figure V-6. *axs2* does not have abnormal responses to the auxin transport inhibitor NPA. Back-crossed *axs2* seeds and wild-type Col-0 seeds were plated on media supplemented with the indicated concentrations of NPA. After eight days of growth, the primary root length was measured. Error bars represent standard errors of the means (*n* ≥ 10). Data supplied by Andrew Woodward.
Figure V-7. *axs2* exhibits a wild-type response to the synthetic auxin 2,4-D. Primary root lengths of eight-day-old back-crossed *axs2* and wild-type seedlings grown in the presence of the indicated concentrations of 2,4-D were measured. Error bars represent standard errors of the means (*n* ≥ 9).
In addition to abnormal gravitropism, \textit{axs2} also has a higher number of lateral roots than wild type. This difference is maintained when the seedlings are grown in conditions that induce lateral root formation such as high temperatures or growth on IBA (Figure V-8). The primary root of the mutant appears longer than the primary root of wild-type seedlings, which suggested that the difference in lateral root number might be due to faster growth rate. However, the primary root of \textit{axs2} is not longer than wild-type's primary root in every condition analyzed, even if \textit{axs2}'s lateral root number is higher than wild-type for that condition (Figure V-9). Moreover, the primary root of \textit{axs2} appears supersensitive to IBA-mediated inhibition of root elongation (Figure V-9). IBA may act as a slow-release form of IAA (Bartel et al. 2001), and low enough concentrations of IAA may induce the same response in \textit{axs2}. In conclusion, these results suggest that the lateral root phenotype observed in \textit{axs2} is due to auxin supersensitivity and not due indirectly to a faster growth rate.

Many of the effects mediated by auxin depend on the balance between auxin and other phytohormones, such as cytokinin or ethylene. In fact, the majority of the auxin resistant mutants that have been isolated exhibit cross-resistance to other phytohormones (see Chapter I). Therefore, it was of interest to determine whether the auxin supersensitive mutant \textit{axs2}, even after being back-crossed, still did not exhibit abnormal responses to other phytohormones. We analyzed the response of \textit{axs2} to cytokinin-mediated inhibition of primary root elongation as well as \textit{axs2}'s response to ethylene. The results for the cytokinin analysis are summarized in Figure V-10, which shows that \textit{axs2} exhibits a wild-type response to cytokinin. In addition, \textit{axs2} exhibits a wild-type
response to ethylene treatment in the dark, where the phytohormone inhibits root and hypocotyl elongation and promotes the formation of an apical hook (data not shown).

**Figure V-8.** *axs2* has a higher number of lateral roots than wild-type. Col-0 wild-type and back-crossed *axs2* mutant five-day old seedlings grown initially on unsupplemented media at 22 °C or 28 °C were transferred to growth media supplemented with auxins as indicated. Growth continued for three more days at the same temperature at which the seedlings were initially grown (indicated on graph), and the number of lateral roots was measured. Error bars represent standard errors of the means (n ≥ 10).
**Figure V-9.** *axs2* is supersensitive to IBA in primary root elongation. The primary root of *axs2* is longer than wild-type primary root only when no auxins are supplied exogenously. Col-0 wild-type and back-crossed *axs2* mutant five-day old seedlings grown initially on unsupplemented media at 22 °C or 28 °C were transferred to growth media supplemented with auxins as indicated and maintained at the same temperature as for the original growth (indicated on graph). The primary root growth that occurred three days after transfer was measured. Error bars represent standard errors of the means (n ≥ 10).
Figure V-10. *axs2* exhibits a wild-type response to the phytohormone cytokinin. Primary root lengths of eight-day-old Col-0 wild-type and *axs2* back-crossed seedlings grown in the presence of the indicated concentrations of cytokinin were measured. Error bars represent standard errors of the means (*n* ≥ 9).
V.C.3. *axs3* is hypersensitive to high temperatures, which induce auxin accumulation

The third mutant isolated as a result of the *axs* screen is *axs3*. The mutation that causes the *axs3* phenotype was originally concealed in the *axs2* mutant background, but it was then separated by crossing from the mutation that causes the *axs2* phenotype. The *axs3* mutant exhibits a remarkable response to high temperatures, which are known to induce auxin accumulation (Gray et al. 1998): instead of the high-temperature promotion of root growth observed for wild-type seedlings (Rogg et al. 2001), *axs3* exhibits a high-temperature inhibition of root growth (Figure V-11). In addition, *axs3*’s hypocotyls are resistant to the growth promoting effects of high-temperatures on hypocotyl elongation (Figure V-11).

To determine whether older *axs3* roots were also temperature sensitive, I grew *axs3* seedlings for five days at 22 °C before transferring them to 28 °C, where the seedlings grew for three additional days. The root growth that occurred after transfer was compared to that of wild-type seedlings grown under the same conditions. The effect on root growth after transferring *axs3* to growth media supplemented with auxins was similarly analyzed. The results of this experiment are shown in Figure V-12; they indicate that high temperature dramatically inhibits *axs3* root elongation and that high temperature slightly promotes wild-type root elongation even in older seedlings. In addition, *axs3* appears supersensitive to IBA, but given that the mutant has a shorter root than wild-type, the significance of this result is difficult to interpret.
Figure V-11. High temperature inhibits root elongation in *axs3* whereas it promotes root elongation in wild-type. Col-0 wild-type and out-crossed *axs3* seeds were grown at 22 °C or at 28 °C to induce auxin accumulation. After nine days the seedlings were removed from the media and photographed. Figure supplied by Andrew Woodward.
Figure V-12. The effect of high temperatures on *axs3* root growth persists in older seedlings. Col-0 wild-type and back-crossed *axs3* mutant five-day old seedlings grown initially on unsupplemented media at 22 °C were transferred to growth media supplemented with auxins as indicated, at either 22 or 28 °C. The primary root growth that occurred three days after transfer was measured. Error bars represent standard errors of the means ($n \geq 6$).
The short root phenotype of *axs3* is dramatically enhanced at high temperatures; therefore, we wanted to determine if this defect is a result of slower overall growth, enhanced at high temperatures, or whether this defect is specific for primary root elongation. To analyze these possibilities, we grew *axs3* seedlings in the absence of hormones for 5 days prior to their transfer to auxins in order to induce lateral root formation. After three days of growth, the number of lateral roots present in each seedling was counted. The results, shown in Figure V-13, indicate that the *axs3* mutation does not result in an overall slower growth because the mutant makes a wild-type number of lateral roots. Therefore, it is reasonable to assume that the mutation has a specific effect on root elongation.

In conclusion, *axs3* is supersensitive to high temperature, which promotes auxin accumulation in wild-type seedlings. High temperature results in an inhibition of *axs3* primary root growth; this response is present in seedlings germinated at 28 °C or in seedlings that are transferred to 28 °C five days after germination. The *axs3* mutant is different from auxin resistant mutants because the latter simply do not respond to high-temperature promotion of hypocotyl elongation (Gray et al. 1998), whereas *axs3* responds to high temperature with a decrease in growth. This is an exciting result because *axs3* may be supersensitive to its own auxin being accumulated as a result of the high temperature, and therefore auxin inhibits *axs3* growth as it inhibits wild-type growth at supraoptimal concentrations. Alternatively, *axs3* may be a temperature-sensitive allele of a gene required for growth. There are known temperature sensitive mutations in cell-wall proteins or structural cellular components that result in abnormal root growth at the non-permissive high temperatures (Arioli et al. 1998; Peng et al. 2000; Hussey and
Hawkins 2001; Lane et al. 2001). However, none of these mutants shares the *axs3* mapping region. It is also possible that this phenotype is related to stress and not specifically to auxin. However, it is still very interesting to understand *axs3*’s response given that high temperatures constitute an environmental stress encountered by many organisms, and temperature can strongly influence agricultural productivity.

![Graph showing number of lateral roots](image)

**Figure V-13. axs3 has the same number of lateral roots as wild-type.** Col-0 wild-type and back-crossed *axs3* mutant seeds were plated on unsupplemented media and allowed to grow for five days. Then, the seedlings were transferred to media containing auxins to induce lateral root formation and allowed to grow for three more days. The number of lateral roots was counted under a dissecting microscope. Error bars represent standard errors of the means (*n* ≥ 6).
**V.D. *axs* mutant mapping**

To further characterize and understand the defects in the *axs* mutants, it is of interest to isolate the gene defective in these mutants. In preparation for cloning, we mapped these mutants using recombination mapping. We used the root-curling defect to map *axs1* and *axs2*, and the hypersensitivity to high temperatures to map *axs3*. The three mutations were mapped to chromosome I. The mapping procedures and results are discussed in this section.

**V.D.1. Molecular mapping of *axs1* and *axs2**

The *axs1* mutant exhibits two basic phenotypes suggestive of altered auxin homeostasis: resistance to low concentrations of auxin transport inhibitors and abnormal root-curling and root-waving behavior. The *axs2* mutant exhibits also a variety of phenotypes that suggest auxin supersensitivity: mainly it has enhanced lateral root formation and defective root-curling and root-waving. When analyzing F₂ plants from a mapping out-cross the root-waving behavior appeared to be the most evident phenotype for both *axs1* and *axs2*, and we chose it to select F₂ plants for mapping purposes. Using PCR-based markers, we analyzed the genotypes of non-curling F₂ plants from an out-cross between *axs1* and Ws, or from an out-cross between *axs2* and Ler. We mapped both *axs1* and *axs2* mutations to a region of chromosome I located between the markers nga111 and nga280 (Figure V-14). We developed additional PCR-based markers to narrow this mapping region. These markers are listed in Table V-2.
Figure V-14. The defect in root-curling of *axs1* and of *axs2* map to the same interval on chromosome I. Non-curling F$_2$ seedlings derived from a cross between *axs1* and Ws, or between *axs2* and Ler, were analyzed using PCR-based polymorphic markers to map the location of the *axs1* and *axs2* mutations. The bars represent chromosome I, and the hatched lines represent the mapping interval of the mutations. To the left of the bars, the name of the polymorphic markers is indicated, and in parenthesis their location in centiMorgans is indicated. The numbers at the right of the bars represent the number of recombinant chromosomes identified over the total number of chromosomes analyzed. Because both mutations map to a similar place, it is possible that this location corresponds to a QTL that is responsible for more relaxed root-curling behavior in the Col-0 ecotype. Data supplied by Andrew Woodward.
Table V-2. New PCR markers developed for mapping *axs* mutants

<table>
<thead>
<tr>
<th>Marker</th>
<th>Enzyme</th>
<th>Size of products (bp)</th>
<th>Oligonucleotides (5'-3' orientation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Col-0</td>
<td>Ler</td>
</tr>
<tr>
<td>F13O11</td>
<td>160</td>
<td>125</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>7G6</td>
<td>340, 90</td>
<td>430</td>
</tr>
<tr>
<td></td>
<td>Accl</td>
<td>179</td>
<td>166</td>
</tr>
<tr>
<td></td>
<td>F27G20</td>
<td>257</td>
<td>219</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>T9L6</td>
<td>308, 122</td>
<td>430</td>
</tr>
<tr>
<td></td>
<td>BglII</td>
<td>200, 360</td>
<td>560</td>
</tr>
<tr>
<td></td>
<td>Alul</td>
<td>178</td>
<td>123, 55</td>
</tr>
<tr>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

These PCR markers were developed with Andrew Woodward. Marker T7G6 was originally developed by J. Bender (personal communication), and only the PCR primers were modified for this study.

The mapping data for *axs1* and *axs2* indicates that both of these mutations map to the same region of chromosome 1, suggesting they might be allelic. However, a complementation test indicated that these mutations are not allelic because F₁ seedlings exhibit wild-type root curling. Further analysis indicated that the Col-0 ecotype generally exhibits more relaxed root-curling than the Ler or Ws ecotypes. That is, although both mutants do exhibit an even more relaxed root-curling phenotype than the parental Col-0 wild-type, both ecotypes to which the mutants were out-crossed for mapping purposes exhibit tighter curling than Col-0. Therefore, it is possible that instead of mapping *axs1* or *axs2*, we mapped a QTL.

It is possible that in order for the *axs1* and *axs2* phenotypes to be apparent in the out-cross two loci are needed: one QTL in chromosome 1 and an additional locus located somewhere else in the genome. However, if this were true, the mutations would map to two positions in the Arabidopsis genome, which is not the case. Alternatively, the
hypothesised two loci could be contained in the interval mapped to chromosome I, which would explain the inability to narrow down the mapping region. It appears that in order to identify the molecular defect in *axis2*, it will be necessary to select a mapping population based on the lateral root phenotype, rather than on the root-curling behavior. If *axis2* is out-crossed to an ecotype that exhibits the same number of lateral roots as wild-type Col-0, the enhanced lateral root behavior may be used for mapping effectively.

**V.E. Molecular mapping of *axis3***

We mapped the *axis3* mutation by analyzing the F$_2$ population derived from an *axis3* to Ler out-cross. We grew the F$_2$ seeds at high temperatures, and selected those with the *axis3* hypersensitivity in root elongation to analyze their genotypes at known PCR-based markers. Andrew Woodward mapped the *axis3* mutation to chromosome I, to a position between the markers nga63 and nga280 (Figure V-15). We developed additional markers to narrow down this mapping region; these are listed in Table V-2. Using these new markers, Andrew Woodward isolated a region of approximately 900 Kb to which the *axis3* mutation maps (Figure V-15). This region is covered by 13 BACs, which have been sequenced and annotated by the AGI (Kaul et al. 2000). However, we did not identify any known genes or auxin-related genes that could be originating the *axis3* phenotype in this region. Therefore, it will be necessary to further narrow down the *axis3* mapping region before identifying the gene defective in the *axis3* mutant.
Figure V-15. \textit{axs3} maps to a region of 900 Kb in chromosome I. The \textit{axs3} mutation was localized to a region of chromosome I contained between the markers nga63 and nga280. To further refine this position, additional PCR-based markers were developed. \textit{axs3} was mapped between the markers F5M6 and 7G6, a region of 900 Kb contained in the indicated sequenced BACs. The bars represent the chromosome with the PCR-based markers indicated on top of them. The numbers under the markers represent the number of recombinant chromosomes identified over the total number of chromosomes analyzed. Data provided by Andrew Woodward.
V.F. Discussion

Because negative regulators of auxin homeostasis are presumed to exist, we reasoned that mutations in the genes important for this regulation would render a plant supersensitive to auxin. I developed a genetic screen to identify these mutants. This screen resulted in the identification of three mutants that appear auxin supersensitive: *axs1*, *axs2*, and *axs3*. In addition, twelve other putative mutants were originally isolated as *axs* mutants. For many of these, the difference in auxin sensitivity observed between mutant and wild-type was not bigger than the difference observed between ecotypes. Because this screen was originally designed to identify genes important for maintenance of auxin homeostasis, the inability to map these twelve putative mutants, and therefore the inability to identify the genes defective in them, resulted in the abandonment of these mutants. However, it is worth pointing out that the fact that I could not identify mutants with dramatic auxin supersensitive root elongation further corroborates that auxin homeostasis is very tightly regulated. The inability may be a reflection of the many pathways that regulate auxin homeostasis, and the manners in which they can cooperate: when one pathway is disturbed, the other pathways may come into play to balance IAA homeostasis.

Still, the *axs* screen resulted in the identification of three mutants: *axs1*, *axs2*, and *axs3*. The *axs1* mutant exhibits abnormal root curling and root waving, behaviors associated with auxin. However, it does not appear to be supersensitive to auxin in root elongation or lateral root formation. Whereas it is possible to isolate out-crossed mutants from a mapping population, it has been impossible to isolate back-crossed mutants. This
inability may reflect the fact that the abnormal root behavior results from at least two loci. It is possible that ecotype differences account for the inability to obtain a backcrossed homozygote mutant. I.e., if the Ws ecotype, used for mapping, were a mutant in one of these two loci, it would be possible to observe an effect of the second mutation segregating in the F2 mapping population.

The axs2 mutant behaves as an auxin supersensitive mutant in that it has a higher number of lateral roots than wild type. Other mutants with high number of lateral roots have been found to contain higher levels of auxin than wild type (Boerjan et al. 1995; Delarue et al. 1998; Barlier et al. 2000). Therefore, it is likely that axs2 phenotype is also related to higher levels of auxin, be it through auxin overproduction or through auxin supersensitivity. In addition, axs2 is supersensitive to the slow-release auxin IBA in primary root elongation.

One surprising but welcomed result from the axs screen was axs3. This mutant is hypersensitive to high temperatures, which induce auxin accumulation (Gray et al. 1998). The high-temperature response may reflect a general hypersensitivity to stress, or a temperature-sensitive allele of a protein required for growth. However, the number of lateral roots of this mutant in the presence of IAA or IBA appears wild-type (Figure V-13). This suggests that the defect is specific for root elongation, and not a reflection of slower overall growth. It would be interesting to determine axs3's response to other types of stress. Nevertheless, given that variations in environmental temperature are one of the many stresses that plants encounter in the wild, it would be of great interest to understand how the high temperature response is mediated. Because there are few reports of plant mutants hypersensitive to high temperatures, axs3 provides a unique
opportunity to study the effect of this environmental strain on plants. Alternatively, \textit{axs3} may represent a temperature-sensitive allele of a gene coding for a cellular component necessary for growth. Further characterization of \textit{axs3} and cloning of the gene defective in this mutant will differentiate between these possibilities and lead to a better understanding of the \textit{axs3} phenotype.
CHAPTER VI. CONCLUSIONS AND PERSPECTIVES

VI.A. IAA conjugates and *ilr2*

Plants actively regulate the ratios of conjugated to free IAA. The majority of IAA is found in the conjugated, less active form. The study of mutants that are resistant to the conjugated form of the hormone but that retain sensitivity to the free form has identified proteins that are important for maintaining this active auxin homeostasis. Through this type of screen, the Arabidopsis amidohydrolases that cleave IAA-amino acid conjugates were identified (Bartel and Fink 1995; Davies et al. 1999), as well as IAR1 (Lasswell et al. 2000). The Arabidopsis mutant *ilr2-1* was isolated as an IAA-Leu resistant mutant that retains wild-type sensitivity to free IAA (B. Bartel, personal communication). This thesis describes the characterization of the *ilr2-1* mutant and the analysis of the gene defective in this mutant.

VI.A.1. *ilr2-1* is resistant to IAA-Leu and IAA-Phe, but not to other IAA conjugates

In addition to being resistant to IAA-Leu, I found that *ilr2-1* is resistant to IAA-Phe, but not to other conjugated forms of IAA (Figure III-1). This result further supports the hypothesis that the different conjugated forms of IAA may serve different functions (Bartel et al. 2001). In addition, *ilr2-1* exhibits other phenotypes independent of the growth media: it has a slower growing root than wild type (Figure III-3) and it is defective in lateral root formation (Figure III-6). Low concentrations of IAA conjugates
supplied exogenously can rescue the lateral root defect of *ilr2-l* (Figure III-6), supporting the hypothesis that *ilr2-l* is defective in IAA homeostasis, which causes the decreased lateral root phenotype.

**VI.A.2. *ilr2-l* exhibits altered metal sensitivity**

The *ilr2-l* mutant is also resistant to cobalt and manganese, but not to other metals including zinc, iron, and copper (Figure III-11). Other IAA-amino acid resistant mutants, including *iar1*, are not resistant to the effects of these metals. This suggests that the *ilr2-l* cobalt resistance is not due to an indirect effect of the IAA-Leu resistance, but rather that ILR2 is important for mediating cobalt, manganese, and IAA-Leu sensitivity.

Interestingly, low concentrations of cobalt added to the growth medium can rescue the short root phenotype of *ilr2-l* (Figure III-14), which suggested that *ilr2-l* might be deficient in cobalt accumulation. However, metal analysis indicates that *ilr2-l* plants grown in the absence of supplied cobalt contain higher levels of cobalt than wild-type plants (Table III-3). In addition to high levels of cobalt, *ilr2-l* mutant plants also accumulate higher levels of manganese and iron than wild-type plants (Table III-3). Paradoxically, the short root phenotype of *ilr2-l* can also be rescued by low concentrations of manganese (Figure III-12B) or zinc but not by iron (Figure III-14). Zinc import into plant cells depends on the same transporters as iron and manganese (Clemens 2001). Therefore, it is possible that supplying low concentrations of cobalt, zinc or manganese can affect iron transport.

In the future, it will be interesting to compare metal transport in *ilr2* mutants and wild type. This experiment would analyze whether the *ilr2* mutations affect metal
sensitivity through an effect on metal transport. In addition, it may shed some light on the mechanisms whereby exogenous cobalt can rescue growth defects of a mutant that contains elevated cobalt levels. More specifically, if ilr2-1 cobalt import is equivalent to wild-type cobalt’s import, then it would be reasonable to speculate that ilr2-1 is resistant to cobalt because it has called into operation specific mechanisms that alleviate the endogenous cobalt accumulation. These mechanisms may result in a perceived reduced concentration of cobalt that can thus be rescued by low concentrations of the metal. Alternatively, cobalt may alleviate ilr2-1’s short root indirectly. Growth of wild type and ilr2-1 seedlings on cobalt results in a reduction in the concentration of manganese, chromium and potassium (Table III-3). The ilr2-1 mutant is resistant to manganese as well as to cobalt (Figure III-11), and both cobalt (Table III-3) and zinc (Clemens 2001) can affect manganese content. Therefore, it is possible that even if the ilr2-1 mutant accumulates equivalent manganese concentrations as wild type, its short root phenotype can be rescued by cobalt and zinc because both of these metals can affect manganese transport.

VI.A.3. The ilr2-2 mutant has opposite phenotypes from ilr2-1

The original ilr2-1 mutant was isolated in a Ws background. I identified a novel ilr2 mutant, ilr2-2, in the Col-0 background. This mutation results from a T-DNA insertion 140 bp upstream from the predicted first ATG, probably representing an allele of ILR2 with reduced function. Interestingly, in contrast to ilr2-1, ilr2-2, exhibits cobalt supersensitivity whereas the mutation has no effect on IAA-Leu sensitivity. This suggests that ilr2-1 represents a gain-of-function allele and ilr2-2 a loss-of-function
allele. This could be confirmed by overexpressing wild-type and ilr2-1 versions of ILR2 in wild-type plants.

VI.A.4. The gene defective in ilr2 contains a predicted ARID DNA binding domain

To better understand the mechanisms by which ILR2 can regulate metal and auxin homeostasis, I cloned the gene defective in the ilr2 mutant using a map-based approach. Using recombination mapping, I localized ILR2 to chromosome III between the markers nga162 and GL1, and the gene defective in the mutant was identified by complementation. The Col-0 form of ILR2 codes for a novel protein with no homology to genes found in the database. ILR2 contains the five strictly conserved amino acids that form the ARID DNA binding domain (Figure IV-4), but because this domain is poorly defined the significance of this homology will have to be determined experimentally. To analyze whether ILR2 can bind DNA it will have to be expressed in a heterologous system that is not dependent on GST-fusion proteins, given that I was unable to use this system to express ILR2 fusion proteins. Members of the ARID protein family bind A/T rich regions, some with sequence specificity and some without sequence specificity. The ILR2 protein can be tested for DNA binding in the non-specific A/T rich regions to which ARID proteins have been found to bind. If ILR2 binds DNA in a sequence-specific manner, the use of DNA chip analysis can be of use. DNA chip analysis can be used to compare the transcripts expressed in ilr2-1, ilr2-2, and wild-type seedlings grown in the presence or absence of cobalt and IAA-Leu. The opposite phenotype presented by ilr2-1 and ilr2-2 would be particularly helpful for this analysis because will allow the distinction of genes that exhibit opposite patterns of expression in these two mutants. A
comparison of the promoters from the genes whose expression is altered in the \textit{ilr2} mutants may identify regions of similarity. These DNA sequences can be tested for ILR2 binding. In addition, DNA chip analysis can also lead to the identification of the genes important for cobalt sensitivity and for this interesting link between metal and auxin homeostasis. However, because the ARID domain is not contained in the short form of \textit{ILR2} it is likely that the short form of ILR2 does not function as a DNA-binding protein.

\textbf{VI.A.5. \textit{ILR2} is polymorphic between Arabidopsis ecotypes}

Upon analysis of the \textit{ILR2} gene, I found that it is polymorphic between Arabidopsis ecotypes and apparently encodes two different proteins in different ecotypes. I analyzed 14 Arabidopsis ecotypes, two contain an \textit{ILR2} isoform that encodes a predicted 147 amino acid protein (Ws and Ler), whereas the other 12 (including Col-0), contain an isoform that encodes a predicted 396 amino acid protein (Figure IV-5). The observation that a mutation in the short \textit{ILR2} can be rescued by the long \textit{ILR2} indicates that both forms of the gene are functional. The short form might be less efficient than the long form, resulting in the higher accumulation of \textit{ILR2} mRNA in Ws compared to Col-0 (Figure IV-10). In fact, wild-type Ws seedlings are more sensitive to cobalt than wild-type Col-0 seedlings (Figure IV-7), which may be another result of the \textit{ILR2} polymorphism. Alternatively, both isoforms of the gene may produce identical proteins whose promoters are different, thereby explaining the difference in \textit{ILR2} mRNA accumulation. Analysis of cDNAs isolated from both types of genes would be helpful in differentiating between these two possibilities. However, the \textit{ILR2} gene is expressed at undetectable levels in the Col-0 ecotype, and I was unable to identify a cDNA from Col-0.
The identified polymorphism spans the region of ILR2 predicted to have the ARID DNA binding domain. It is tempting to speculate that the short ILR2 is functional by interacting with a DNA binding domain located in a different part of the Ws genome. However, sequence inspection of the partially completed Ler sequence database (www.tigr.org) did not identify a translocation of the 983 bp missing from the short ILR2, and repeated PCR efforts have failed to amplify a product from the deleted region from Ws or Ler DNA. Therefore, if the short ILR2 is functioning by interacting with another DNA binding domain, this might represent a novel interaction.

The AGI has sequenced the Col-0 accession DNA (Kaul et al. 2000), and a variety of private and public efforts are aimed at analyzing the genome of other ecotypes. The identified polymorphism for ILR2 suggests that by comparing the genomes of the different Arabidopsis ecotypes, it might be possible to identify essential parts of novel proteins.

VI.A.6. Ions and IAA conjugate sensitivity

The combined effects of the ilr2 mutations on metal and auxin homeostasis suggests two competing hypotheses: ilr2 could affect cobalt homeostasis and indirectly affect the sensitivity to some IAA-amino acid conjugates, or ILR2 could be important for some aspect of auxin conjugate sensitivity that is directly related to metal homeostasis.

Several lines of evidence suggest a tight relationship between metal homeostasis and phytohormone regulation (see Chapter I). Of particular interest is the fact that the amidohydrolases are dependent on metal cofactors to mediate IAA-amino acid hydrolysis. The availability of these cofactors may serve a regulatory mechanism for auxin homeostasis, supporting the hypothesis that ilr2 indirectly affects IAA-Leu sensitivity through its effects on metal homeostasis. Interestingly, the two metals to
which \textit{ilr2-1} exhibits resistance, cobalt and manganese, are the two metals that the amidohydrolases can use as \textit{in vitro} cofactors to mediate IAA-amino acid hydrolysis (LeClere et al. 2001). However, the \textit{ilr2-1} mutation only affects IAA-Leu sensitivity and not IAA-Ala sensitivity, suggesting that if the \textit{ilr2-1} mutation affects conjugate sensitivity as an indirect effect from altered cobalt or manganese homeostasis, this effect must be specific for IAA-Leu without having any effects on the IAA-Ala response.

The second hypothesis, that ILR2 is important for some aspect of auxin conjugate sensitivity directly related to metal homeostasis, assumes that cobalt and/or manganese sensitivity are tightly linked to auxin sensitivity. Unfortunately, not much is known about the effects of cobalt or manganese in plants or about the mechanisms important for the homeostasis of these metals. Experiments like the proposed DNA chip analysis on mutant \textit{ilr2} and wild-type plants grown with or without cobalt, and analysis of the metal transport capabilities of \textit{ilr2}, might help analyze if this hypothesis is correct.

First, the genes whose expression is altered in wild-type plants exposed to cobalt could be identified. The expression of these genes would then be analyzed in the \textit{ilr2} mutants grown with or without cobalt. In addition, the genes important for IAA-Leu sensitivity could be identified by analyzing the gene expression of wild-type plants exposed to IAA-Leu and comparing this group to mutants resistant to IAA-Leu, such as \textit{ilr2-1} and \textit{ilr1}. The availability of the \textit{ilr2-2} presumed null allele that exhibits supersensitivity to cobalt may be helpful to determine which genes are of particular interest, by comparing those transcripts whose behavior is opposite in \textit{ilr2-1} from \textit{ilr2-2}. Finally, the group of genes identified as important for cobalt sensitivity could be compared to the group of genes identified as important for IAA-Leu sensitivity. The
analysis of *ilr2*’s metal transport would analyze whether *ilr2*-*l*’s resistance to cobalt results form reduced sensitivity to exogenous cobalt, or whether it results from enhanced cobalt accumulation leading to acclimation to specific ranges of cobalt concentrations. These two studies would help define whether a real link exists between metal and auxin conjugate homeostasis, and if ILR2 is important for this connection.

Cobalt is an essential ion to all forms of life. When cobalt is not present in soil, the use of cobalt-containing fertilizers is necessary, especially if the soils are used for ruminant feeding (Hamilton 1994). Deficiency in cobalt in soils used for ruminant feeding results in vitamin B-12 deficiency in those animals (Hamilton 1994). *ilr2* is the only known plant mutant with altered cobalt sensitivity. Therefore, these mutants may be particularly useful to understand the mechanisms used by plants to sense and/or respond to cobalt.

**VI.B. Auxin supersensitive mutants**

Several lines of evidence suggest the existence of negative regulators of auxin homeostasis and response:

I. The ubiquitin-dependent degradation pathway is important for proper auxin responses (Leyser et al. 1993; Ruegger et al. 1998; Gray et al. 2001), suggesting that negative regulators of auxin action must be degraded in order for the auxin response to be mounted.

II. Mutations that stabilize the auxin-inducible *AU1IAA* genes result in aberrant auxin responses. Some *Aux/IAA* mutants are resistant to auxin (Nagpal et al. 2000; Reed 2001; Rogg et al. 2001) whereas others are supersensitive to auxin in
some tissues and resistant in others (Rouse et al. 1998). Because the stabilization of these proteins causes aberrant phenotypes, it can be inferred the degradation pathway must be important for the auxin response.

III. Mutants that overproduce auxin rarely accumulate free IAA (Normanly et al. 1993; Ouyang et al. 2000); the excessive IAA is usually conjugated to other moieties. In addition, supplying Arabidopsis seedlings with high concentrations of radioactive IAA results in the formation of high concentrations of unusual IAA species (Slovin et al. 1999). This suggests that upon excessive accumulation of IAA, several mechanisms important for decreasing the levels of active auxin are activated.

VI.B.1. Design and results of the axs mutant screen

I designed and conducted a genetic screen to isolate auxin supersensitive (axs) mutants. The axs screen was designed specifically to identify mutants with a supersensitive response to auxin-mediated inhibition of primary root elongation. I identified a group of 14 mutants with axs phenotypes. Eight of these mutants exhibited supersensitivity in root elongation and lateral root formation, and four mutants exhibited only a lateral root phenotype. However, after back-crossing or out-crossing these 14 mutants, some of them lost their phenotype indicating that the mutation was not behaving as a single heritable trait. Other mutations behaved as single heritable traits in back-crossed populations, but the difference in auxin sensitivity observed between mutant and wild-type was not larger than the difference between ecotypes. Because this screen was originally designed to identify genes important for maintenance of auxin homeostasis, the
inability to map these mutants, and therefore the inability to identify the genes defective in these mutants, resulted in the elimination of 12 out of the 14 identified mutants. The inability to identify more \( axs \) mutants may reflect the dynamic nature of auxin homeostasis: if one pathway is disturbed, other pathways can come into play to alleviate impending auxin accumulation.

**VI.B.2. \( axs1 \) and \( axs2 \) exhibit abnormal root gravitropic behaviors**

The \( axs \) screen resulted in the identification of three \( axs \) mutants: \( axs1 \), \( axs2 \) and \( axs3 \). \( axs1 \) and \( axs2 \) were originally identified in the primary screen because of an enhanced number of lateral roots. Though I was not able to recapitulate this phenotype in subsequent generations of \( axs1 \), the \( axs2 \) mutant still exhibits enhanced number of lateral roots after being back-crossed. In addition to the lateral root phenotypes, \( axs1 \) and \( axs2 \) exhibit abnormal root-curling and root-waving behaviors. Both of these root phenotypes are thought to result from thigmotropic and gravitropic stimuli, in which auxin is known to play an important role. Using the abnormal root-waving behavior, both the \( axs1 \) and \( axs2 \) mutations were mapped to chromosome I, between the markers nga280 and nga111. The mapping region for \( axs1 \) spans 18 cM, which contains the 15 cM mapping region for \( axs2 \). The F1 seedlings from a complementation cross between \( axs1 \) and \( axs2 \) exhibit wild-type root-curling, indicating that these mutations are not allelic. Despite further attempts, the mapping region for \( axs1 \) or \( axs2 \) could not be further refined.

I was not able to identify \( axs1 \) back-crossed mutants in a segregating F2 population despite being able to identify \( axs1 \) out-crossed mutants. This suggests that the \( axs1 \) phenotype is not due to a single mutation. It is possible that the \( axs1 \) phenotype is due to
two mutations in the Col-0 background, and Ws, the ecotype to which the *axs*/* mutant was out-crossed for mapping purposes, is a mutant for one of these two loci.

The Col-0 ecotype, in which both mutations were isolated, exhibits more relaxed root-curling than the Ws or Ler ecotypes, to which the mutants were out-crossed. Therefore, it is possible that when analyzing the F$_2$ seedlings generated from these out-cresses, the individuals selected for root-curling phenotype carry a Col-0 QTL responsible for the more relaxed root-curling. However, F$_2$ individuals selected from both crosses exhibit a more relaxed root-curling phenotype than either one of the wild-type parental lines. This suggests two possibilities. First, the segregating phenotypes could be originated by two loci, one of which is a QTL. However, neither the *axs*/* nor the *axs*/* mutations mapped to two regions on the Arabidopsis genome. Therefore, if two loci are responsible for the phenotypes, the mutation and the QTL must map to closely related regions in chromosome I, which would explain the inability to further narrow down the mapping region. The second possibility is that the QTL becomes stronger in the presence of a non-Col-0 locus, which would explain why the identified F$_2$ individuals exhibit more relaxed root-curling than either of the parental wild-type lines.

Despite the difficulties with *axs*/*, the *axs*/* mutant does exhibit true auxin-supersensitive phenotypes in the back-crossed line, including enhanced lateral root formation. If this phenotype were used for mapping purposes instead of the root-curling phenotype, it will indicate whether the location mapped to chromosome I corresponds to a QTL. The use of the enhanced lateral root formation phenotype would help identify the genetic defect responsible for the auxin supersensitivity, for which the *axs* screen was originally designed.
VI.B.3. *axs3* is supersensitive to high temperatures

The third mutant isolated from the *axs* screen, *axs3*, exhibits supersensitivity to high temperature in root elongation. High temperature promotes auxin accumulation, and auxin resistant mutants do not respond to hypocotyl-growth induction by high temperatures (Gray et al. 1998). Temperature-sensitive alleles of genes necessary for primary cellular functions result in high-temperature mediated inhibition of root growth. It is possible that *axs3* represents this type of mutant. Interestingly, *axs3* also appears supersensitive to the slow-release auxin IBA. The mutant exhibits a slightly shorter root than wild-type even when grown in conditions that do not induce auxin accumulation. However, this phenotype is not due to overall slower development because *axs3* makes as many lateral roots as wild-type, suggesting both reach equivalent developmental stages at the same time.

Using the high-temperature phenotype, *axs3* was mapped to a 900 Kb region on chromosome 1 between the markers nga63 and nga280. This region contains 10 BACs that have been fully sequenced by the AGI. However, sequence examination did not identify any known auxin-related genes that could be responsible for the phenotype. Therefore, it will be necessary to further narrow down this region before identifying the gene defective in the *axs3* mutant.

It is possible that the defect in the *axs3* mutant is not directly related to auxin, but rather it is related to high temperatures as a form of environmental stress. To distinguish between these possibilities, the response of *axs3* to other characterized environmental stresses can be assessed, including the response to osmotic stress and low temperatures. Because *axs3* has a similar number of lateral roots as wild type, it is possible that the
shorter root is due to smaller cells, in which case their shape and organization in the root can be analyzed.

Independent of whether the *axs3* mutant phenotype is due to a response to environmental stress or to a response to auxin, it will be of great interest to isolate the gene defective in this mutant. Auxin supersensitivity is not well characterized, and understanding how high temperature promotes auxin responses, and how the high-temperature response is mediated, would undoubtedly aid in our understanding of auxin homeostasis. High temperatures affect many aspects of agriculture and understanding how plants are able to respond to this stress will be both interesting and useful.
CHAPTER VII. REFERENCES


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