RICE UNIVERSITY

Genes, Organelles, and Molecules that Influence Plant Development through Auxin Regulation

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

Humankind depends on plants to harvest solar energy and convert it into accessible chemical energy. With booming human population growth and diminishing availability of arable land, understanding plant development is necessary for more efficient agricultural production. Auxin is a plant hormone utilized in many aspects of plant growth and environmental responses. This work examines genes that regulate or are regulated by auxin, biogenesis and function of an organelle that is an auxin source, and molecules that behave as auxins to influence plant development.

Within the plant cell, peroxisomes are organelles that house many processes including fatty acid metabolism to produce energy and also proto-auxin metabolism to produce the active hormone. Peroxisomal proteins are translated in the cytoplasm and imported into peroxisomes by a host of machinery. Peroxisomal targeting signal sequences are recognized by one of two receptors; these receptors interact with each other physically and functionally in some organisms. Here, I identify the receptor machinery present in diverse organisms to predict and compare methods of peroxisomal matrix protein import. I also characterize mutants of the model plant Arabidopsis thaliana defective in import of one class of peroxisomal matrix proteins.

In addition, I examine various molecules that influence plant development in an auxin-like fashion. I identify genes with mRNA accumulation regulated by a proto-auxin in a background that inefficiently converts this compound into auxin. I describe the characterization of responses to a second auxin-related molecule that impacts plant development through auxin signaling. I also describe the isolation, characterization, and cloning of a mutant with reduced sensitivity to a specific subset of auxin-like molecules.

Data obtained in this work reveal a host of factors that affect auxin regulation and thereby influence plant life. The results of these experiments in plant biology highlight the diversity, complexity, and essentiality of auxin responses.
Acknowledgements

My years in the Bartel lab have been the best of my life. It is a difficult place to leave, and I will not yet depart. First and foremost, I thank Bonnie Bartel for her thoughtful consideration, sharing her vast knowledge without a hint of condescension. She is a hugely interested and active leader, and she makes the research process facile and efficient. Bonnie’s focus on the big picture and simultaneous attention to detail is a dual-focus perspective I hope to emulate.

Mónica Magidin taught me how to do science. As an undergraduate researcher in the Bartel lab, I was very green and unsure. She took a student accustomed to highminded theory and put the practices of routine experimentation into that perspective. Like Bonnie, Mónica is able to think about efficiency and good practice while constantly musing about theory.

As a graduate student, undergraduates were extremely helpful to me. My sister Erin Woodward performed an indole-3-propionic acid mutant screen described in Chapter 6, the output of which is a current focus of work. Erin Woodward, Arthur Millius, Gretchen Troxler, and Jean Bao performed some genotypic analyses of peroxin mutants described in Chapter 4.

I will remember my coworkers in the Bartel lab with gratitude and respect. In addition to Mónica, Bethany Zolman, Rebekah Rampey, and Melanie Monroe-Augustus were particularly helpful in my ‘experimental education’ and fostered a collaborative spirit. I also thank Jeanne Rasbery and Diana Dugas for collaborative work and sharing ideas. In addition, thanks to Dave Nelson, Jamie Lasswell, Luise Rogg, Dereth Phillips, and Lucia Strader for thoughtful advice and frequent scientific discussions.

Finally, thanks to my committee for consideration and shared wisdom. Janet Braam, James McNew, Fred Rudolph, George Bennett, and David Queller were conscientious advisors.
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<tr>
<td>2,4-D</td>
<td>2,4-dichlorophenoxyacetic acid</td>
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<tr>
<td>A</td>
<td>adenine</td>
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<td>ABA</td>
<td>abscisic acid</td>
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<td>ABRC</td>
<td>Arabidopsis Biological Resource Center</td>
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<td>ACC</td>
<td>1-amino-cyclopropane-1-carboxylic acid</td>
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<td>AMP</td>
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<td>BLAST</td>
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<tr>
<td>IAAld</td>
<td>indole-3-acetaldehyde</td>
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Chapter 1: Background and significance*

To fully understand auxin regulation, action, and interactions will be to understand many aspects of plant growth and development. As a critical plant hormone, auxin modulates such diverse processes as tropic responses to light and gravity, general root and shoot architecture, organ patterning, vascular development, and growth in tissue culture (Davies, 1995). The importance of auxin for human sustenance is both vital and readily apparent: auxin is required for plant growth. Anthropogenic manipulation of auxin physiology has assisted plant propagation, and, through the blind pressure of artificial selection, the development of modern crop varieties (Multani et al., 2003; Salamini, 2003).

Auxin biology is among the oldest fields of experimental plant research. Charles Darwin performed early auxin experiments, observing the effects of a hypothetical substance modulating plant shoot elongation to allow tropic growth toward light (Darwin, 1880). Darwin’s experiments expanded upon Theophil Ciesielski’s research examining roots bending toward gravity (Ciesielski, 1872). The term auxin was coined by scientists examining plant growth-modulating substances in human urine named auxins A and B (Kögl and Haagen Smit, 1931). A structurally distinct compound with auxin activity isolated from fungi was called heteroauxin; auxins A and B were gradually abandoned for the reproducibly bioactive heteroauxin, which was later determined to be indole-3-acetic acid (IAA; Thimann, 1977).

1.A. Compounds with auxin activity

Because auxins influence virtually every aspect of plant growth and development, numerous bioassays for auxin response have been described. These assays have proven useful in the isolation of endogenous auxins, the identification of auxin precursors, and

* Portions of this chapter have been published previously (Woodward and Bartel, 2005b).
the development of synthetic auxin-like compounds (Thimann, 1977). One of the earliest noted auxin effects was in phototropism, the curvature of stems toward a light source (Darwin, 1880). Application of auxin to decapitated shoots can induce such bending in the absence of a light stimulus (Went, 1926), and several nonphototropic mutants are deficient in auxin signaling components (Harper et al., 2000; Tatematsu et al., 2004).

The pea curvature test also employs auxin-regulated differential growth: dark-grown (etiolated) Pisum sativum stems are decapitated, sliced along part of their length, and floated in solution containing compounds being tested (Fawcett et al., 1960; Wain and Wightman, 1954). In auxin solution, stem segments bend inward, while in water they curl outward (Went and Thimann, 1937). Other tests to establish whether a given compound exerts auxin-like effects include spraying tomato plants and application to wheat coleoptiles, where auxin causes characteristic stem bending and elongation, respectively (Fawcett et al., 1960; Wain and Wightman, 1954).

Another early assay for auxin activity was in tissue culture, where auxins promote rooting from undifferentiated callus (Skoog and Miller, 1957). Along with the phytohormone cytokinin, which induces shoot formation, auxin allows regeneration of plants from cultured callus (Krikorian, 1995).

Current assays for auxin response in the model plant Arabidopsis thaliana often involve growth of seedlings on medium supplemented with the compound of interest. Auxins profoundly influence root morphology, inhibiting root elongation, increasing lateral root production (Figure 1.1), and inducing adventitious roots (Zimmerman and Hitchcock, 1942). The relevance of these bioassays to normal plant physiology is supported by the observation that mutants that overproduce auxin tend to have abundant lateral and adventitious roots, along with long hypocotyls and petioles, and epinastic leaves and cotyledons (Boerjan et al., 1995; Delarue et al., 1998; King et al., 1995; Zhao et al., 2001). Conversely, mutants deficient in auxin responses are often characterized by long primary roots, few lateral roots, and short hypocotyls when grown on
Figure 1.1. Auxins promote lateral root formation and inhibit root elongation. *Arabidopsis thaliana* Col-0 ecotype plants were grown on unsupplemented medium (Haughn and Somerville, 1986) for six days, then transferred to unsupplemented medium (A) or medium supplemented with 10 nM IAA (B), 100 nM 2,4-D (C), 100 nM NAA (D), or 10 μM IBA (E) and grown for six additional days. F, Plants were grown on various concentrations of natural and synthetic auxins for eight days. Points represent means +/- standard error, n ≥ 8. All plants were grown at 22 °C under yellow light.
unsupplemented medium in the light, in addition to reduced auxin responses in the bioassays described above (Estelle and Somerville, 1987; Hobbie and Estelle, 1995; Monroe-Augustus et al., 2003).

Many naturally-occurring compounds that exert auxin-like effects have been revealed by these bioassays (Figure 1.1). IAA, an extensively-studied endogenous auxin, is active in all bioassays described above and is often potent at nanomolar concentrations (Figure 1.1). A chlorinated form of IAA with high auxin activity, 4-Cl-IAA, is found in several plants (Slovin et al., 1999). In addition to the indolic auxins, phenylacetic acid (PAA) has been identified in plants and is an active auxin (Ludwig-Müller and Cohen, 2002; Wightman, 1977).

Certain IAA precursors, such as indole-3-acetonitrile and indole-3-pyruvic acid, are also active in bioassays, presumably because of conversion in the tissue to IAA (Thimann, 1977). Similarly, indole-3-butyric acid (IBA), identical to IAA except for two additional methylene groups in the side chain, is effective in bioassays. Like IAA, exogenous IBA inhibits Arabidopsis root elongation (Zolman et al., 2000) and induces lateral (Zolman et al., 2000) and adventitious (King and Stimart, 1998) root formation. IBA, originally classified as a synthetic auxin, is in fact an endogenous plant compound (Bartel et al., 2001; Epstein and Ludwig-Müller, 1993; Ludwig-Müller, 2000). IBA is more effective than IAA at lateral root induction, perhaps because, unlike IAA, IBA efficiently induces lateral roots at concentrations that only minimally inhibit root elongation (Zolman et al., 2000); IBA is employed commercially for this purpose (Hartmann et al., 1990a). Biochemical analyses in a variety of plants and genetic studies in Arabidopsis indicate that IBA acts primarily through conversion to IAA in a process resembling peroxisomal fatty acid β-oxidation (Bartel et al., 2001), though roles for IBA independent of conversion to IAA have been proposed (Ludwig-Müller, 2000; Poupart and Waddell, 2000).
Two main types of synthetic plant growth regulators with auxin-like activity have been described: 1-naphthalacetic acid (NAA) and 2,4-D-related compounds. Both compounds exert auxin-like influences, including root elongation inhibition and lateral root promotion (Figure 1.1). The NAA isomer 2-NAA has little activity in bioassays (Thimann, 1977) and provides a weak acid control for auxin experiments employing the active 1-NAA. 2,4-dichlorophenoxybutyric acid (2,4-DB) is a 2,4-D derivative with two additional methylene groups in the side chain (analogous to the structural relationship between IBA and IAA) that elicits similar responses to those observed after 2,4-D treatment. In general, 2,4-dichlorophenoxyacetic acid (2,4-D) and IAA derivatives with even-numbered carbon side chains have more activity than derivatives with odd-numbered carbon side chains (Fawcett et al., 1960; Wain and Wightman, 1954). This result suggests that a process such as β-oxidation could remove two-carbon units from the side chains, arriving at the active acetate form if the substrate started with an even carbon number (Fawcett et al., 1960; Wain and Wightman, 1954). 2,4,5-trichlorophenoxybutyric acid (2,4,5-TB) also exerts auxin-like activity; the infamous defoliant herbicide Agent Orange was a mixture of 2,4-D and 2,4,5-TB (Fallon et al., 1994). Agent Orange was particularly toxic because of dioxin produced as a byproduct of 2,4,5-TB synthesis (Courtney et al., 1970; Schwetz et al., 1973). Today, 2,4-D alone is a widely used herbicide. In addition to NAA and 2,4-D, several alkylated and halogenated forms of IAA exert auxin-like growth responses in various bioassays (Antoli´c et al., 1996; Nigovi´c et al., 2000). Though IAA, 2,4-D, NAA, and other synthetic compounds can cause similar physiological responses in bioassays, the molecules cause distinct but overlapping changes in gene expression (Pufky et al., 2003), perhaps reflecting differences in metabolism, transport, or interaction with the signaling machinery.
1.B. IAA biosynthetic pathways

Arabidopsis seedlings can synthesize IAA in leaves, cotyledons, and roots; young leaves have the highest biosynthetic capacity (Ljung et al., 2001). Although it is widely accepted that plants use several pathways to synthesize IAA, none of the pathways is yet defined to the level of knowing each relevant gene, enzyme, and intermediate. Plant genes implicated in IAA biosynthesis are listed in Table 1.1, and the reactions catalyzed by the encoded enzymes are illustrated in Figure 1.2. Plants use both tryptophan (Trp)-dependent and Trp-independent routes to synthesize IAA; several Trp-dependent pathways have been suggested. Multiple IAA biosynthetic pathways may contribute to regulation of IAA production, but the paucity of informative loss-of-function mutations in IAA biosynthetic enzymes, coupled with functional redundancy, have limited analysis of pathway control and prevented definitive determination of the importance of each pathway. For example, arabidopsis seedlings grown at high temperature accumulate free IAA (Gray et al., 1998) and display high-auxin phenotypes (Gray et al., 1998; Rogg et al., 2001), but the source of the excess IAA is unknown.

1.B.1. Trp-dependent IAA biosynthesis

Several Trp-dependent pathways, which are generally named after an intermediate, have been proposed: the indole-3-pyruvic acid (IPA) pathway, the indole-3-acetamide (IAM) pathway, the tryptamine pathway, and the indole-3-acetaldoxime (IAOx) pathway. An arabidopsis enzymatic complex that converts Trp to IAA in vitro has been partially purified (Müller and Weiler, 2000a), and future biochemical and genetic dissection of the process is likely to reveal the relative importance of the pathways discussed below.

The IPA pathway [Trp → IPA → indole-3-acetaldehyde (IAAld) → IAA] is important in some IAA-synthesizing microorganisms (Koga, 1995) and may operate in
<table>
<thead>
<tr>
<th>Gene</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>IAAld oxidase</td>
<td>cytoplasm</td>
<td>OE in sur1</td>
<td>(Sekimoto et al., 1998; Seo et al., 1998)</td>
</tr>
<tr>
<td>AMII</td>
<td>IAM hydrolase</td>
<td>not reported</td>
<td>not reported</td>
<td>(Pollmann et al., 2003)</td>
</tr>
<tr>
<td>CYP79B2, CYP79B3</td>
<td>monooxygenases</td>
<td>chloroplast</td>
<td>LOF: cyp79B2 cyp79B3: low glucosinolates, IAN, and IAA; OE: resistant to Trp analogs; high indolic glucosinolate, IAN, and IAA-X levels</td>
<td>(Zhao et al., 2002)</td>
</tr>
<tr>
<td>CYP83B1/ SUR2</td>
<td>P450 monooxygenase</td>
<td>cytoplasm</td>
<td>LOF: high IAA, IAAld, and IAA-Asp levels; normal IAN levels; low indolic glucosinolate levels; altered Trp biosynthetic gene expression; defective photomorphogenesis in red light; OE: high indolic glucosinolate levels, reduced apical dominance</td>
<td>(Bak et al., 2001; Barlier et al., 2000; Delarue et al., 1998; Hoecker et al., 2004; Smolen and Bender, 2002)</td>
</tr>
<tr>
<td>NIT1</td>
<td>nitrilase</td>
<td>not reported</td>
<td>LOF: IAN resistant, normal IAA levels</td>
<td>(Normanly et al., 1997)</td>
</tr>
<tr>
<td>NIT2</td>
<td>nitrilase</td>
<td>not reported</td>
<td>OE: increased sensitivity to IAN, normal IAA levels</td>
<td>(Normanly et al., 1997)</td>
</tr>
<tr>
<td>ZmNIT2</td>
<td>nitrilase (maize)</td>
<td>not reported</td>
<td>not reported</td>
<td>(Park et al., 2003)</td>
</tr>
<tr>
<td>SUR1/ RTY/ ALF1/ HLS3</td>
<td>C-S lyase</td>
<td>not reported</td>
<td>LOF: high IAA and IAA-X levels, low glucosinolates</td>
<td>(Boerjan et al., 1995; Celenza et al., 1995; Golparaj et al., 1996; King et al., 1995; Lehman et al., 1996; Mikkelsen et al., 2004)</td>
</tr>
<tr>
<td>TDC</td>
<td>Trp decarboxylase (C. roseus)</td>
<td>cytoplasm</td>
<td>OE: enhanced root curling</td>
<td>(De Luca et al., 1989; Guillet et al., 2000)</td>
</tr>
<tr>
<td>TSA1/TRP3</td>
<td>Trp synthase a</td>
<td>chloroplast</td>
<td>LOF: high IAA-X, IAN, indole-glycerol phosphate, and indolic glucosinolate levels; normal free IAA; low Trp; Trp auxotroph</td>
<td>(Müller and Weiler, 2000b; Normanly et al., 1993; Ouyang et al., 2000)</td>
</tr>
<tr>
<td></td>
<td>Trp synthase b</td>
<td>chloroplast</td>
<td>LOF: high IAA-X and IAN levels, normal free IAA, low Trp; Trp auxotroph</td>
<td>(Normanly et al., 1993; Ouyang et al., 2000)</td>
</tr>
<tr>
<td>ORP</td>
<td>Trp synthase b</td>
<td>chloroplast</td>
<td>LOF: high IAA-X, normal free IAA, Trp auxotroph</td>
<td>(Wright et al., 1992; Wright et al., 1991)</td>
</tr>
<tr>
<td>YUCCA, YUCCA2</td>
<td>FMO-like</td>
<td>cytoplasm</td>
<td>LOF: no phenotype; OE: high IAA levels</td>
<td>(Zhao et al., 2001)</td>
</tr>
<tr>
<td>FLOOZY</td>
<td>FMO-like (petunia)</td>
<td>not reported</td>
<td>LOF: defective leaf venation and apical dominance; OE: high IAA levels</td>
<td>(Tobeña-Santamaria et al., 2002)</td>
</tr>
</tbody>
</table>

* Listed genes are from arabidopsis unless otherwise noted.
Figure 1.2. Potential pathways of IAA biosynthesis in Arabidopsis. 
De novo IAA biosynthetic pathways initiate from Trp or Trp precursors. Compounds that have been quantified in Arabidopsis are in blue, enzymes for which the arabidopsis genes have been cloned are in red, and arabidopsis mutants are in lower case italics. Suggested conversions for which genes have not been identified are indicated with question marks. Trp biosynthesis and the P450-catalyzed conversion of Trp to IAOx are chloroplastic, whereas many Trp-dependent IAA biosynthetic enzymes are apparently cytoplasmic. See Table 1.1 for references.
plants as well (Cooney and Nonhebel, 1991). IPA is found in arabidopsis seedlings (Tam and Normanly, 1998), but genes encoding a Trp aminotransferase that oxidatively transaminates Trp to IPA or an IPA decarboxylase that converts IPA to IAAld have not been identified in plants. The final enzyme in the proposed IPA pathway is an IAAld-specific aldehyde oxidase protein (AAO1) that has increased activity in the IAA-overproducing *superroot1* (*sur1*) mutant (Seo et al., 1998). The identification of arabidopsis AAO1 does not verify the existence of the IPA pathway, however, as IAAld may be an intermediate in other IAA biosynthetic pathways (see below).

The IAM pathway [Trp → IAM → IAA] is a second microbial pathway that also may act in plants. In *Agrobacterium tumificiens* and *Pseudomonas syringae*, for example, Trp monoxygenase (IaAM) converts Trp to IAM, and an IAM hydrolase (IaAH) converts IAM to IAA (Patten and Glick, 1996). IAM lacks auxin activity in arabidopsis, which allows the *iaaH* gene to be used as a screenable marker that confers IAM sensitivity (Brusslan et al., 1993). Intriguingly, IAM is found in arabidopsis seedlings at levels similar to free IAA (Pollmann et al., 2002), and an arabidopsis amidohydrolase (AMI1) converts IAM to IAA *in vitro* (Pollmann et al., 2003). It will be interesting to learn whether disruption of *AMI1* or *AAO1* decreases IAA levels.

**1.B.2. YUCCA may catalyze a rate-limiting step in a tryptamine pathway**

A tryptamine (TAM) pathway [Trp → TAM → *N*-hydroxyl-TAM → indole-3-acetaldoxime (IAOx) → IAAld → IAA] could also convert Trp to IAA (Figure 1.2). Trp decarboxylase converts Trp to tryptamine in the first committed step in the biosynthesis of *Catharanthus roseus* monoterpenoid indole alkaloids (Facchini et al., 2000). The arabidopsis genome contains potential Trp decarboxylase genes, but the encoded enzymes have not been characterized, and tryptamine has not been identified in arabidopsis.
The identification of *yucca*, an IAA-accumulating mutant with classic high-auxin phenotypes (Zhao *et al.*, 2001), suggests that a tryptamine IAA biosynthetic pathway may operate in some plants. *yucca* is resistant to toxic Trp analogs, suggesting that the accumulating IAA is Trp-derived (Zhao *et al.*, 2001). The *yucca* phenotype derives from overexpression of a flavin monooxygenase (FMO)-like enzyme that oxidizes tryptamine to N-hydroxyl-tryptamine *in vitro* (Zhao *et al.*, 2001). The homologous *Petunia x hybrida* enzyme FLOOZY is defective in a mutant deficient in leaf venation and apical dominance (Tobeña-Santamaria *et al.*, 2002). Although the loss-of-function floozy mutant has wild-type IAA levels, overexpressing FLOOZY results in increased IAA levels in shoot apices and young leaves (Tobeña-Santamaria *et al.*, 2002). YUCCA may be a rate-limiting enzyme in the tryptamine pathway, but a test of this hypothesis is hampered by genetic redundancy. Arabidopsis has a family of ten YUCCA-like enzymes, and insertional mutations in YUCCA and YUCCA2 confer no morphological phenotypes (Zhao *et al.*, 2001). The N-hydroxyl-tryptamine produced by YUCCA could be dehydrogenated to IAOx or dehydrogenated and hydrolyzed to IAAld (Figure 1.2). Enzymes that catalyze these conversions have not been identified.

1.B.3. **Indole-3-acetaldoxime is a precursor to indolic glucosinolates that can be converted to IAA**

The IAOx pathway [Trp → IAOx → IAN or IAAld → IAA] is of particular interest in plants like arabidopsis that make indolic glucosinolate secondary metabolites (Fahey *et al.*, 2001), because IAOx is the branchpoint between indole-3-methylglucosinolate and IAA biosynthesis (Figure 1.2). Two arabidopsis P450 monooxygenases, CYP79B2 and CYP79B3, oxidize Trp to IAOx *in vitro* (Hull *et al.*, 2000; Mikkelsen *et al.*, 2000). CYP79B2 overexpressors have increased IAA, IAN (Zhao *et al.*, 2002), and indolic glucosinolate levels (Mikkelsen *et al.*, 2000). Conversely, the cyp79B2 cyp79B3 double mutant has morphological phenotypes suggestive of low auxin, reduced IAA in certain growth conditions, lowered IAN levels, and no detectable indolic.
glucosinolates (Zhao et al., 2002). Taken together, these results are consistent with IAOx serving as a precursor that can be shunted to either auxin or indolic glucosinolates.

A third P450 monooxygenase, CYP83B1, converts IAOx to its N-oxide, the first committed step in indole-3-methylglucosinolate biosynthesis (Figure 1.2; Bak et al., 2001). Loss-of-function cyp83b1 alleles were independently isolated in screens for high-auxin seedling phenotypes (superroot2 or sur2; Delarue et al., 1998), altered resistance to toxic Trp analogs (Smolen and Bender, 2002), defective photomorphogenesis in red light (Hoecker et al., 2004), and P450 monooxygenase insertional disruptions (Winkler et al., 1998). The sur2/cyp83B1 mutant accumulates free IAA (Barlier et al., 2000; Delarue et al., 1998) and the IAA precursor IAAl (Barlier et al., 2000). This phenotypic analysis, along with the nature of the defective gene, suggests that IAOx accumulates in the mutant and is converted to IAAl, which is oxidized to IAA (Figure 1.2).

The surl mutant (Boerjan et al., 1995), also isolated as rooty (King et al., 1995), alfl (Celenza et al., 1995), and hookless3 (Lehman et al., 1996), provides another link between high auxin and defects in glucosinolate production. This mutant has high-auxin phenotypes resembling sur2 and yucca, and accumulates free IAA and IAA conjugates (Boerjan et al., 1995; King et al., 1995; Lehman et al., 1996). surl is defective in a C-S lyase that apparently cleaves S-(indolylacetohydroximoyl)-L-cysteine to indole-3-thiohydroximate, the third step in glucosinolate production from IAOx (Golparaj et al., 1996; Mikkelsen et al., 2004). Indeed, indolic glucosinolates are undetectable in surl (Mikkelsen et al., 2004). Given the multiplicity of available pathways to modulate IAA levels, it is intriguing that arabidopsis plants cannot adequately compensate for the increased IAA precursor levels that result when indolic glucosinolate production is dampened.
1.B.4. Indole-3-acetonitrile and nitrilases in IAA biosynthesis

Nitrilases that can hydrolyze IAN to IAA are found in several plant families, including crucifers and grasses (Thimann and Mahadevan, 1964). These enzymes are encoded by the arabidopsis NIT genes (Bartel and Fink, 1994; Bartling et al., 1992; Bartling et al., 1994) and Zea mays (maize) ZmNIT2 (Park et al., 2003). NIT1 and NIT2 can hydrolyze IAN applied to plants (Normanly et al., 1997; Schmidt et al., 1996), and an enzymatic complex with nitrilase immunoreactivity converts Trp to IAA in vitro (Müller and Weiler, 2000a). IAN is present in arabidopsis (Illic´ et al., 1996; Normanly et al., 1993) and maize (Park et al., 2003), suggesting that this conversion could contribute to IAA homeostasis. In the Brassica, IAN is formed following myrosinase-catalyzed indole-3-methylglucosinolate hydrolysis, and IAN levels tend to track with indolic glucosinolate levels in arabidopsis mutants (Mikkelsen et al., 2000; Müller and Weiler, 2000b; Normanly et al., 1993; Reintanz et al., 2001; Zhao et al., 2002), consistent with nitrilases acting downstream of glucosinolates in arabidopsis. However, IAN has also been suggested as an intermediate in IAOx to IAA conversion (Figure 1.2), although enzymes catalyzing the conversion of IAOx to IAN have not been isolated, and the source of IAN in maize, which lacks indolic glucosinolates, is unknown.

NIT1 is the most highly expressed of the four arabidopsis NIT genes (Bartel and Fink, 1994). nit1 mutants are resistant to exogenous IAN (Normanly et al., 1997), but lack obvious low-auxin phenotypes, indicating that any role played by NIT1 in IAA biosynthesis is redundant. The NIT2 gene is normally expressed at a low level, but is induced by a bacterial pathogen (Bartel and Fink, 1994), by Plasmodiophora (Grsic-Rausch et al., 2000), during arabidopsis leaf senescence (Quirino et al., 1999), and in response to IAN treatment (Grsic et al., 1998). NIT2 induction correlates with decreased IAN levels and increased IAA levels during senescence (Quirino et al., 1999), increased IAA levels in Plasmodiophora-infected roots (Grsic-Rausch et al., 2000), and higher nitrilase immunoreactivity (Müller and Weiler, 2000b) in the IAN-accumulating trp3
mutant (Normanly et al., 1993). NIT3 expression is induced by sulfur starvation, and is correlated with reduced indolic glucosinolate levels and lateral root proliferation (Kutz et al., 2002). Expression of maize nitrilase ZmNIT2 is elevated in embryonic tissue (Park et al., 2003). Upgrading these correlations between expression and IAA levels to causal relationships awaits the analysis of additional nit family mutants and would be aided by an arabidopsis nit1 nit2 nit3 triple mutant.

1.B.5. Analyses of trp mutants reveal Trp-independent IAA biosynthesis

In addition to the proposed Trp-dependent IAA biosynthetic pathways (Figure 1.2), analyses of Trp biosynthetic mutants demonstrate that plants also can synthesize IAA without using a Trp intermediate. The arabidopsis trp3-1 and trp2-1 mutants are defective in Trp synthase α and β, respectively (Last et al., 1991; Radwanski et al., 1996). These mutants accumulate amide- and ester-linked IAA conjugates (Normanly et al., 1993; Ouyang et al., 2000), despite having low soluble Trp levels (Müller and Weiler, 2000b; Ouyang et al., 2000). Similarly, the maize orange pericarp Trp synthase b mutant accumulates IAA conjugates (Wright et al., 1992; Wright et al., 1991). Unlike trp2 and trp3, plants blocked earlier in the Trp pathway, such as trp1 (Last and Fink, 1988) and antisense plants with decreased indole-3-glycerol phosphate synthase (IGS) levels, do not accumulate IAA conjugates (Normanly et al., 1993; Ouyang et al., 2000).

Analyses of the trp mutants imply that a Trp-independent IAA biosynthetic pathway branches from indole-3-glycerol phosphate or indole (Figure 1.2). Trp synthase a and b normally channel indole-3-glycerol phosphate to Trp without indole release. In maize, however, Trp synthase a-like enzymes can act without b subunits to produce indole released as a volatile or converted into certain defense compounds (Frey et al., 1997; Frey et al., 2000; Melanson et al., 1997) or possibly IAA. Arabidopsis contains two apparent Trp synthase a genes: TSA1, the gene defective in the trp3 mutant (Radwanski et al., 1996), and a second uncharacterized gene (At4g02610).
Because IAA conjugates are hydrolyzed under alkaline conditions (Baldi et al., 1989; Bialek and Cohen, 1986), total (free plus conjugated) IAA is often inferred without knowledge of the conjugates present by quantifying free IAA after alkaline hydrolysis. The specificity of the alkaline hydrolysis evidence used to support the importance of the Trp-independent pathway has been questioned (Müller and Weiler, 2000b). Application of this technique requires accommodation for the indolic biochemistry of the plant under study. For example, IAN, which is present in arabidopsis, is hydrolyzed to IAA under alkaline conditions, so IAN must be separately quantified and subtracted from apparent total IAA values (Ilic’ et al., 1996). As the individual conjugates of arabidopsis are identified and quantified, it will be interesting to learn the precise conjugate profiles in the various trp mutants, and to reinvestigate alkaline-releasable IAA in mutant plants that lack indolic glucosinolates, for example.

An independent method to clarify biosynthetic pathways involves feeding plants isotopically-labeled substrates, which, in a linear pathway, will result in isotopic enrichment of a precursor relative to its product. Intact arabidopsis seedlings do not efficiently convert \(^{[\text{H}_3]}\text{-Trp}\) into IAA, but the Trp precursor \(^{[\text{N}]}\text{-anthranilate}\) labels IAA more completely than Trp (Normanly et al., 1993), confirming the importance of Trp-independent IAA biosynthesis during normal growth. Arabidopsis shoot and root explants, however, do efficiently convert \(^{[\text{H}_3]}\text{-Trp}\) to IAA (Müller et al., 1998b; Müller and Weiler, 2000b). Because the explant process may damage tissue, this result suggests that Trp-dependent IAA biosynthesis may be wound-induced in arabidopsis, as it is in bean (Sztein et al., 2002). Plants may switch from basal Trp-independent IAA biosynthesis to Trp-dependent pathways during stress, when more IAA may be needed (Ribnicky et al., 2002; Sztein et al., 2002). Studies examining metabolism of a recently synthesized, isotopically-labeled indole may allow dissection of Trp-independent IAA biosynthesis (Ilic’ and Cohen, 2004).
1.B.6. IAA Storage: Conjugates and indole-3-butyric acid

Higher plants can store IAA in the form of IAA conjugates and indole-3-butyric acid (IBA), which can provide free IAA upon hydrolysis or β-oxidation, respectively (Figure 1.3). IAA can be ester-linked to sugars or amide-linked to amino acids and peptides. Proposed functions for these conjugates include storage, transport, compartmentalization, excess IAA detoxification, and protection against peroxidative degradation (Cohen and Bandurski, 1982). Certain IAA conjugates are active in auxin bioassays, and several plants store IAA conjugates in seeds that are hydrolyzed during germination to provide free IAA to developing seedlings. In contrast, biologically inactive conjugates present in plants are probably intermediates in IAA degradation. Analyses of arabidopsis mutants defective in various facets of IAA homeostasis are revealing the roles of the diverse IAA sources during plant growth and development.

1.B.7. IAA conjugate identification and functions

Different plant species have distinct IAA conjugate profiles (Cohen and Bandurski, 1982; Slovin et al., 1999). Experiments using alkaline hydrolysis to release free IAA from conjugates indicate that arabidopsis maintains ~90% of IAA in amide linkages, with an additional ~10% as ester-linked conjugates and ~1% as free IAA (Normanly et al., 1993; Tam et al., 2000). Low levels of IAA-Ala, IAA-Asp, IAA-Glu, and IAA-Leu are present in arabidopsis seeds (Rampey et al., 2004) and seedlings (Kowalczyk and Sandberg, 2001; Rampey et al., 2004; Tam et al., 2000). However, most of the amide-linked conjugates in arabidopsis seeds are solvent insoluble (Ljung et al., 2002), suggesting that single-amino acid conjugates constitute only part of the amide fraction in this tissue. A 35 kDa IAA-peptide is present in arabidopsis seeds; the large size of this conjugate may contribute to the solvent insolubility of amide conjugates (Ljung et al., 2002). Although genes encoding arabidopsis IAA-peptides have not been identified, an IAA-modified bean protein is similar to a soybean late seed maturation
Figure 1.3. Potential pathways of IAA metabolism.
Compounds that have been quantified in Arabidopsis are in blue, enzymes for which the Arabidopsis genes have been cloned are in red, and Arabidopsis mutants are in lower case italics. Suggested conversions for which plant genes have not been identified are indicated with question marks. A family of amidohydrolases that apparently reside in the ER lumen can release IAA from IAA conjugates. IIIR1 has specificity for IAA-Leu (Bartel and Fink, 1995), whereas IAR3 prefers IAA-Ala (Davies et al., 1999). Maize (Zm) iaglu and Arabidopsis UGT84B1 esterify IAA to glucose (Jackson et al., 2001; Szerszen et al., 1994); the enzymes that form and hydrolyze IAA-peptides have not been identified. IBA is likely to be converted to IAA-CoA in a peroxisomal process that parallels fatty acid β-oxidation to acetyl-CoA (Bartel et al., 2001).
protein (Walz et al., 2002), suggesting that certain seed storage proteins may function in both amino acid and phytohormone storage. In addition to amide conjugates, the ester conjugate IAA-glucose has also been quantified in several dicotyledonous plants (including Arabidopsis) and the monocot maize (Jakubowska and Kowalczyk, 2004; Tam et al., 2000).

Among divergent plant phyla, endogenous IAA, IAA-amide, and IAA-ester levels are quite variable (Sztein et al., 1999). The lycophyte Selaginella kraussiana accumulates large quantities of conjugates, particularly IAA-amide compounds (Sztein et al., 1999). After feeding labeled IAA to the lycophyte S. kraussiana, the fern Ceratopteris richardii, and various mosses and liverworts, varied species-specific conjugate profiles become apparent; the conjugates formed include both previously-identified and unknown IAA conjugates (Sztein et al., 1999). These results suggest ancient roles for conjugates in plant biology.

IAA-amino acid conjugates found in plants can be classified into two groups based on bioassay activity and susceptibility to hydrolysis in planta or by plant enzymes. IAA-Ala and IAA-Leu efficiently inhibit Arabidopsis root elongation and are substrates of Arabidopsis amidohydrolases (Bartel and Fink, 1995; Campanella et al., 2003; Davies et al., 1999; LeClere et al., 2002; Rampey et al., 2004). In Arabidopsis, IAA-Ala is present at highest levels in shoots, whereas IAA-Leu accumulates in roots (Kowalczyk and Sandberg, 2001), but neither conjugate is formed at detectable levels following IAA application to seedlings or leaves (Barratt et al., 1999; Östin et al., 1998). These results suggest that IAA-Ala and IAA-Leu function to supply free IAA.

In contrast, although IAA-Asp and IAA-Glu also are present in Arabidopsis (Kowalczyk and Sandberg, 2001; Tam et al., 2000), they are not appreciably hydrolyzed by Arabidopsis seedlings (Östin et al., 1998), and are inefficient inhibitors of root elongation (Campanella et al., 1996; LeClere et al., 2002). Tissues such as expanding leaves and roots that contain the highest free IAA levels also contain the highest levels of
IAA-Asp and IAA-Glu (Kowalczyk and Sandberg, 2001). These results are consistent with an IAA catabolic role for IAA-Asp and IAA-Glu (see “IAA Inactivation” section).

1.B.8. Genetic analysis of IAA conjugate hydrolysis

Several mutant screens using different bioactive IAA-amino acid conjugates have been conducted. If conjugates with auxin activity function solely through free IAA release, then conjugate-resistant mutants that retain wild-type sensitivity to IAA may have defects in conjugate uptake or hydrolysis. If bioactive conjugates play additional roles, these also may be uncovered through mutant analyses. *ilrl* was isolated as an IAA-Leu resistant mutant with reduced sensitivity to root elongation inhibition caused by exogenous IAA-Leu. *ilrl* is defective in an amidohydrolase that cleaves IAA-Leu and IAA-Phe (Bartel and Fink, 1995). Similarly, *iar3* is IAA-Ala resistant and is defective in an amidohydrolase homologous to ILR1 that specifically hydrolyzes IAA-Ala (Davies *et al.*, 1999). The ILR1-like protein ILL2 is the most active IAA amidohydrolase *in vitro* (LeClere *et al.*, 2002); however, no *ill2* alleles were isolated in genetic screens for conjugate-resistant root elongation. Though *ILR1* and *IAR3* are expressed in seedling roots, *ILL2* appears to be expressed predominantly in the shoot (Rampey *et al.*, 2004). An *ill2* T-DNA allele is sensitive to IAA-Leu, IAA-Phe, and IAA-Ala, but, when combined in double and triple mutants with *ilrl* and *iar3*, *ill2* contributes to IAA-Phe resistance in roots and hypocotyls and IAA-Ala resistance in hypocotyls (Rampey *et al.*, 2004).

Interestingly, *ilrl iar3 ill2* triple mutant seedlings display reductions in lateral root number, hypocotyl elongation in the light, sensitivity to exogenous IAA, and free IAA levels (Rampey *et al.*, 2004). These results suggest that the endogenous IAA conjugate substrates of these hydrolases (IAA-Ala and IAA-Leu) are physiologically relevant sources of free IAA. The IAA-Leu insensitivity of the *ilrl iar3 ill2* mutant implies that at least some IAA conjugates with auxin activity act solely via their
hydrolysis to free IAA. However, the triple hydrolase mutant retains partial responsiveness to IAA-Ala (Rampey et al., 2004), suggesting that IAA-Ala has some hydrolysis-independent activity or that additional enzymes hydrolyzing IAA-Ala remain to be discovered.

The iar1 mutant is resistant to the known substrates of the ILR1 and IAR3 amidohydrolases and is defective in a membrane protein (Lasswell et al., 2000) that weakly resembles the ZIP family of metal transporters (Guerinot, 2000). Although the substrate and membrane localization of IAR1 are unknown, the fact that the amidohydrolases require divalent cations such as Mn$^{2+}$, Co$^{2+}$, or Cu$^{2+}$ for activity in vitro (Bartel and Fink, 1995; Davies et al., 1999; LeClere et al., 2002) suggests that metal homeostasis could impact conjugate hydrolysis by modulating amidohydrolase activity. Further supporting a role for metal homeostasis in IAA conjugate metabolism, the IAA-Leu and IAA-Phe resistant ilr2 mutant is also resistant to exogenous Co$^{2+}$ and Mn$^{2+}$ (Magidin et al., 2003). Because the novel ILR2 protein appears to influence metal transport and the ilr2 mutant has a conjugate resistance profile similar to iar1, ILR2 may indirectly affect IAA-conjugate metabolism by negatively regulating transport of metals that influence ILR1 activity (Magidin et al., 2003).

The IAA-Ala resistant mutant iar4 harbor a defective mitochondrial-type pyruvate dehydrogenase E1α (LeClere et al., 2004). iar4 is generally defective in root elongation, but is resistant to several IAA-amino acid conjugates. Although a direct role for pyruvate dehydrogenase in IAA-conjugate hydrolysis is difficult to envision, the slight resistance of iar4 to the synthetic auxin 2,4-D implies that the mutant may be generally deficient in auxin metabolism or response. It is possible that pyruvic acid itself, or an anabolic or catabolic product, influences IAA homeostasis. Alternatively, a complex including IAR4 may function directly in IAA biosynthesis, catalyzing indole-3-pyruvic acid dehydrogenation to yield IAA-CoA, a hypothetical precursor of IAA or IAA conjugates (LeClere et al., 2004).
The genes defective in the icr1 (IAA-conjugate resistant), icr2 (Campanella et al., 1996), and ilr3 (RA Rampey, M Tierney, and B Bartel, unpublished data) mutants have not been reported. Genes currently implicated in IAA-conjugate responses are listed in Table 1.2. Because ilr2, ilr3, iar4, icr1, and icr2 are each represented by a single allele isolated in forward genetic screens, it is likely that conjugate resistance screens are not yet saturated. Sequence analysis suggests that the IAA-amino acid conjugate hydrolases reside in the ER lumen (Bartel and Fink, 1995; Davies et al., 1999). Interestingly, the essential auxin binding protein ABP1 (Chen et al., 2001) is also predominantly ER-localized (Jones, 1994), reinforcing the possibility of a role for this compartment in auxin biology. Analysis of additional mutants may reveal genes required for conjugate import into or IAA efflux from the ER, amidohydrolase transcript accumulation, or amidohydrolase localization, activity, or stability. In theory, conjugate-resistant mutants that fail to import conjugates from the medium might be isolated as well (see “Auxin Transport” section).

1.B.9. The endogenous auxin IBA is converted to IAA in peroxisomes

IBA is a naturally-occurring auxin in a variety of plants (Bartel et al., 2001; Epstein and Ludwig-Müller, 1993; Ludwig-Müller, 2000). Arabidopsis seedlings contain somewhat less free IBA than IAA (Ludwig-Müller et al., 1993), although detailed studies indicating whether this trend holds at all developmental stages have not been completed. Conditions that change IAA levels tend to similarly alter IBA levels (Ludwig-Müller et al., 1993), suggesting that IAA and IBA metabolism are linked. Indeed, arabidopsis seedlings fed labeled IAA make labeled IBA, suggesting that IBA is synthesized from IAA (Ludwig-Müller and Epstein, 1994). Because IBA also acts as an IAA precursor (see below), IBA could function similarly to bioactive IAA conjugates in IAA homeostasis (Bartel et al., 2001).

The auxin activity of IBA results, at least in part, from its conversion to IAA (Figure 1.3). Isolation and characterization of arabidopsis mutants with IBA-resistant,
Table 1.2. Plant genes implicated in IAA conjugate metabolism.

<table>
<thead>
<tr>
<th>Gene*</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>ILR1</td>
<td>IAA-amino acid amidohydrolase</td>
<td>ER lumen</td>
<td>LOF: IAA-Leu resistant</td>
<td>(Bartel and Fink, 1995)</td>
</tr>
<tr>
<td>IAR3</td>
<td>IAA-amino acid amidohydrolase</td>
<td>ER lumen</td>
<td>LOF: IAA-Ala resistant</td>
<td>(Davies et al., 1999)</td>
</tr>
<tr>
<td>TaIAR3</td>
<td>IBA-Ala amidohydrolase (wheat)</td>
<td>not reported</td>
<td>not reported</td>
<td>(Campanella et al., 2004)</td>
</tr>
<tr>
<td>ILL1</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>IAA-amino acid amidohydrolase</td>
<td>ER lumen</td>
<td>LOF: enhances IAA-amino acid conjugate resistance of ilr1 and iar3</td>
<td>(Bartel and Fink, 1995; Rampey et al., 2004)</td>
</tr>
<tr>
<td>DFL1/ GH3-6</td>
<td>IAA-amino acid synthase</td>
<td>not reported</td>
<td>OE: IAA resistant, few lateral roots, dwarf, short hypocotyl in light</td>
<td>(Nakazawa et al., 2001; Staswick et al., 2002)</td>
</tr>
<tr>
<td>YDK1/ GH3-2</td>
<td>IAA-amino acid synthase</td>
<td>not reported</td>
<td>OE: dwarf, few lateral roots, de-etiolated</td>
<td>(Staswick et al., 2002; Takase et al., 2004)</td>
</tr>
<tr>
<td>IAR1</td>
<td>putative ZIP family transporter</td>
<td>membrane</td>
<td>LOF: IAA-amino acid conjugate resistant</td>
<td>(Lasswell et al., 2000)</td>
</tr>
<tr>
<td>ILR2</td>
<td>novel protein</td>
<td>not reported</td>
<td>LOF: IAA-Leu resistant</td>
<td>(Magidin et al., 2003)</td>
</tr>
<tr>
<td>IAR4</td>
<td>pyruvate dehydrogenase E1α subunit</td>
<td>mitochondrion</td>
<td>LOF: IAA-Ala resistant</td>
<td>(LeClere et al., 2004)</td>
</tr>
<tr>
<td>ICR1</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>not reported</td>
<td>not reported</td>
<td>IAA-Phe resistant</td>
<td>(Campanella et al., 1996)</td>
</tr>
<tr>
<td>ILR3</td>
<td>not reported</td>
<td>not reported</td>
<td>IAA-Leu resistant</td>
<td>(RA Rampey, M Tierney, S Leibovich, and B Bartel, unpublished data)</td>
</tr>
<tr>
<td>UGT84B1</td>
<td>IAA glucosyl-transferase</td>
<td>not reported</td>
<td>OE: IAA resistant, reduced apical dominance</td>
<td>(Jackson et al., 2002; Jackson et al., 2001)</td>
</tr>
<tr>
<td>iaglu</td>
<td>IAA glucosyl-transferase (maize)</td>
<td>not reported</td>
<td>not reported</td>
<td>(Szerszen et al., 1994)</td>
</tr>
<tr>
<td>IAMT1</td>
<td>IAA-methyl transferase</td>
<td>not reported</td>
<td>not reported</td>
<td>(Zubieta et al., 2003)</td>
</tr>
</tbody>
</table>

* Listed genes are from Arabidopsis unless otherwise noted.
IAA-sensitive root elongation is clarifying our understanding of IBA action (Poupard and Waddell, 2000; Woodward and Bartel, 2005a; Zolman and Bartel, 2004; Zolman et al., 2001a; Zolman et al., 2001b; Zolman et al., 2000). Mutants with specific β-oxidation defects are IBA resistant, suggesting that IBA is converted to IAA in a process paralleling fatty acid β-oxidation. Because plants β-oxidize fatty acids solely in peroxisomes (Gerhardt, 1992; Kindl, 1993), and several IBA-response mutants also have peroxisomal defects, IBA to IAA conversion is likely peroxisomal.

Peroxisomal β-oxidation of seed storage lipids provides energy to germinating seedlings in oil-seed plants like Arabidopsis. As a result, Arabidopsis fatty acid utilization mutants require supplemental sucrose after germination to prevent developmental arrest (Hayashi et al., 1998). Similarly, many IBA-response mutants are sucrose-dependent during seedling development, have reduced rates of seed storage lipid utilization, and are IBA resistant in both root elongation and lateral root initiation (Woodward and Bartel, 2005a; Zolman and Bartel, 2004; Zolman et al., 2001a; Zolman et al., 2001b; Zolman et al., 2000). These phenotypes suggest defects in the peroxisomal β-oxidation of long-chain fatty acids and IBA. Other IBA-response mutants appear to metabolize long-chain fatty acids normally during germination (Adham et al., 2005; Zolman et al., 2000), but may still have defects in IBA β-oxidation, perhaps due to lesions in isozymes specific to short-chain substrates and IBA.

Cloning the genes defective in several IBA-response mutants (Table 1.3) has substantiated the essential role of peroxisomal β-oxidation in IBA activity. In addition to the proteins required directly in peroxisomal metabolism, more than 20 proteins are required for peroxisome biogenesis and import of peroxisomal matrix proteins from the cytoplasm (Mullen et al., 2001; Olsen, 1998; Subramani, 1998; Tabak et al., 1999). Mutations in PEX5 or PEX7, receptors that bind and transport proteins into the peroxisomal matrix (Olsen, 1998; Subramani, 1998), confer IBA-response defects (Woodward and Bartel, 2005a; Zolman et al., 2000). pex5 and pex7 likely have defects
<table>
<thead>
<tr>
<th>Gene</th>
<th>Product</th>
<th>Putative Localization</th>
<th>Mutant Phenotypes*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACX1</td>
<td>long chain acyl-CoA</td>
<td>peroxisome</td>
<td>IBA resistant, sucrose independent</td>
<td>(Adham et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>oxidase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACX3</td>
<td>medium chain acyl-CoA</td>
<td>peroxisome</td>
<td>IBA resistant, sucrose independent</td>
<td>(Adham et al., 2005; Eastmond et al., 2000b)</td>
</tr>
<tr>
<td></td>
<td>oxidase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACX4</td>
<td>short chain acyl-CoA</td>
<td>peroxisome</td>
<td>IBA resistant, 2,4-DB resistant, sucrose</td>
<td>(Adham et al., 2005; Rylott et al., 2003)</td>
</tr>
<tr>
<td></td>
<td>oxidase</td>
<td></td>
<td>independent</td>
<td></td>
</tr>
<tr>
<td>AIM1</td>
<td>multifunctional protein</td>
<td>peroxisome</td>
<td>abnormal inflorescence meristems, IBA resistant,</td>
<td>(Richmond and Bleecker, 1999; Zolman et al., 2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>sucrose dependent</td>
<td></td>
</tr>
<tr>
<td>PED1</td>
<td>thiolase</td>
<td>peroxisome</td>
<td>IBA resistant, sucrose dependent</td>
<td>(Hayashi et al., 1998; Zolman et al., 2000)</td>
</tr>
<tr>
<td>PXA1</td>
<td>ABC transporter-like</td>
<td>peroxisome</td>
<td>IBA resistant, sucrose dependent, fewer lateral</td>
<td>(Zolman et al., 2001b)</td>
</tr>
<tr>
<td></td>
<td>roots</td>
<td></td>
<td>roots</td>
<td></td>
</tr>
<tr>
<td>PEX5</td>
<td>receptor for peroxisomal</td>
<td>peroxisome</td>
<td>IBA resistant, weak sucrose dependence</td>
<td>(Brickner et al., 1998; Zolman et al., 2000)</td>
</tr>
<tr>
<td></td>
<td>matrix protein import</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEX6</td>
<td>ATPase</td>
<td>peroxisome</td>
<td>IBA resistant, sucrose dependent, fewer lateral</td>
<td>(Zolman and Bartel, 2004)</td>
</tr>
<tr>
<td></td>
<td>roots</td>
<td></td>
<td>roots</td>
<td></td>
</tr>
<tr>
<td>PEX7</td>
<td>receptor for peroxisomal</td>
<td>peroxisome</td>
<td>IBA resistant, sucrose independent; strong</td>
<td>(Woodward and Bartel, 2005a)</td>
</tr>
<tr>
<td></td>
<td>matrix protein import</td>
<td></td>
<td>sucrose dependence and fewer lateral roots in</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>pex7-1 pex5-1</em> double mutant</td>
<td></td>
</tr>
<tr>
<td>PEX14/</td>
<td>docking protein for</td>
<td>peroxisome</td>
<td>IBA resistant, sucrose dependent</td>
<td>(Hayashi et al., 2000; Hayashi et al., 1998;</td>
</tr>
<tr>
<td>PED3</td>
<td>PEX5</td>
<td></td>
<td></td>
<td>Monroe-Augustus, 2004)</td>
</tr>
<tr>
<td>IBR1</td>
<td>not reported</td>
<td>not reported</td>
<td>IBA resistant, sucrose independent</td>
<td>(Zolman et al., 2000)</td>
</tr>
<tr>
<td>IBR3</td>
<td>not reported</td>
<td>not reported</td>
<td>IBA resistant, sucrose independent</td>
<td>(Zolman et al., 2000)</td>
</tr>
</tbody>
</table>

*All mutants are presumed loss-of-function alleles.
importing β-oxidation enzymes from the cytoplasm, slowing β-oxidation and causing IBA resistance. Another IBA-response mutant is defective in the peroxisome biogenesis gene *PEX6* and has abnormal peroxisome morphology (Zolman and Bartel, 2004). PXA1, a membrane protein that is ~30% identical to human and yeast ATP-binding cassette transporters implicated in importing long-chain fatty acids into peroxisomes (Dubois-Dalcq *et al.*, 1999; Holland and Blight, 1999), is defective in another IBA-response mutant (Zolman *et al.*, 2001b). Because *pxa1* is resistant to IBA and is sucrose-dependent during seedling development, PXA1 is probably necessary for the import of both IBA and fatty acid (or the corresponding CoA esters) into peroxisomes (Footitt *et al.*, 2002; Hayashi *et al.*, 2002; Zolman *et al.*, 2001b).

Defects in β-oxidation enzymes can also lead to IBA resistance (Table 1.3). Several arabidopsis peroxisomal β-oxidation defective mutants have been isolated using resistance to the IBA analog 2,4-dichlorophenoxybutyric acid (2,4-DB; Hayashi *et al.*, 1998), which is converted to the active synthetic auxin 2,4-D similarly to IBA β-oxidation (Wain and Wightman, 1954). 2,4-DB resistant mutants include *acx3* (Eastmond *et al.*, 2000b), *acx4* (Rylott *et al.*, 2003), *aim1* (Richmond and Bleecker, 1999), and *ped1* (Hayashi *et al.*, 1998), which are also IBA resistant (Adham *et al.*, 2005; Zolman *et al.*, 2000). *acx* mutants have defects in acyl-CoA oxidases catalyzing the second step of fatty acid β-oxidation, *aim1* (*abnormal inflorescence meristem*) is a mutant in a multifunctional protein acting in the third and fourth steps of fatty acid β-oxidation (Richmond and Bleecker, 1999), and *peroxisome defective 1* (*ped1*) is defective in a thiolase catalyzing the final step of β-oxidation (Hayashi *et al.*, 1998). Moreover, mutations in the gene encoding PEX14/PED2, which docks PEX5 at the peroxisome membrane, confer resistance to 2,4-DB (Hayashi *et al.*, 2000; Hayashi *et al.*, 1998) and IBA (Monroe-Augustus, 2004).

Because arabidopsis mutants defective in fatty acid β-oxidation enzymes and peroxisome biogenesis proteins are IBA resistant, IBA likely is converted to IAA in
peroxisomes. It remains to be determined whether enzymes that catalyze fatty acid β-oxidation also directly catalyze IBA β-oxidation, or whether there are peroxisomal enzymes dedicated to IBA β-oxidation. At least some fatty acid β-oxidation enzymes appear not to act on IBA, as evidenced by the normal IBA and 2,4-DB responses of the *lacs6 lacs7* double mutant, which is sucrose dependent due to defects in peroxisomal acyl-CoA synthetases catalyzing the first step of fatty acid β-oxidation (Fulda *et al*., 2004). If IBA to IAA conversion requires dedicated enzymes, one would expect to recover IBA-response mutants defective in these isozymes that retain normal fatty acid β-oxidation. Moreover, the inferred peroxisomal localization of IBA to IAA conversion implies the existence of a hydrolase that releases IAA from the CoA ester (Figure 1.3), unless this thioester is efficiently hydrolyzed nonenzymatically, and a transporter that effluxes IAA or IAA-CoA out of the peroxisome. Indeed, several sucrose-independent IBA-response mutants, including *ibr1* and *ibr3*, are candidates for having defects in such functions (Zolman *et al*., 2000).

Several peroxisome defective IBA-response mutants have reduced lateral root initiation not only following IBA exposure (Zolman *et al*., 2000), but also in the absence of exogenous auxin. Similarly, certain *Pyrus communis* (pear) plants with lateral root formation defects apparently do not convert IBA to IAA (Baraldi *et al*., 1993). These defects imply that the IAA formed from endogenous IBA β-oxidation during seedling development is important for lateral root initiation. The lateral rooting defects in the peroxisome defective IBA-response mutants are more severe than those of the conjugate hydrolase triple mutant (Rampey *et al*., 2004), suggesting that conjugate hydrolysis does not fully compensate for a lack of IBA β-oxidation, and vice versa.

A few IBA-response mutants with apparently normal fatty acid β-oxidation are less sensitive than wild type to the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) and auxin transport inhibitors (Zolman *et al*., 2000). The *rib1* (resistant to IBA) mutant is in this class (Poupard and Waddell, 2000). Moreover, the *Lateral rootless*
(Lrt1) *Oryza sativa* (rice) mutant is resistant to IAA, IBA, and 2,4-D in terms of root elongation, but only IBA can restore lateral root initiation to the mutant (Chhun *et al.*, 2003). Identifying the genes defective in these IBA-response mutants may reveal IAA-independent roles for IBA or unique features of IBA biology, such as factors differentially mediating IBA and IAA transport (Rashotte *et al.*, 2003).

Like IAA, much of the IBA in plants is conjugated to other moieties through amide- and ester-linkages (Epstein and Ludwig-Müller, 1993; Ludwig-Müller, 2000). A wheat homolog of the arabidopsis IAR3 IAA-Ala hydrolase is inactive on IAA conjugates, but rather hydrolyzes amino acid conjugates of IBA including IBA-Ala, which is present in wheat extracts (Campanella *et al.*, 2004). It will be interesting to learn whether the other members of the monocot amidohydrolase family have specificity for IAA- or IBA-amino acid conjugates. In arabidopsis, IBA is largely ester linked (Ludwig-Müller *et al.*, 1993), suggesting that a different family of enzymes will catalyze IBA release. Although the complete IAA and IBA conjugate profiles have not been reported for any plant, it is likely that these profiles will be complex and reflect the diversity and specificities of the corresponding conjugate hydrolases and synthases.

### 1.C. IAA Inactivation

Pathways that inactivate IAA (Figure 1.3) counteract the inputs to the IAA pool. As discussed above, IAA conjugates that accumulate following exposure of arabidopsis to IAA apparently differ from those used for IAA storage, consistent with the conjugated moiety dictating the fate of the attached IAA (Cohen and Bandurski, 1982). Arabidopsis permanently inactivates applied IAA by ring oxidation to oxIAA (Figure 1.3), which can then be conjugated to hexose (Östlin *et al.*, 1998). In addition, IAA is conjugated to Asp and Glu after applying 5 μM IAA (Östlin *et al.*, 1998), and to Asp, Glu, Gln, and glucose in response to 500 μM IAA (Barratt *et al.*, 1999). Arabidopsis seedlings do not appreciably hydrolyze IAA-Asp and IAA-Glu, and IAA-Asp can be further oxidized to
oxIAA-Asp (Östin et al., 1998), reinforcing the catabolic nature of Asp conjugation. The
catabolic conjugation system is probably present during normal growth, because IAA-
Asp and IAA-Glu are present at low levels in Arabidopsis seedlings (Kowalczyk and
Sandberg, 2001; Rampey et al., 2004; Tam et al., 2000).

In response to elevated IAA levels, catabolic conjugation pathways may be up-
regulated and storage conjugation pathways down-regulated. For example, the sur2
mutant accumulates free IAA (see above) and IAA-Asp (Barlier et al., 2000), an
intermediate in permanent IAA inactivation (Normanly, 1997; Slovin et al., 1999).
However, sur2 plants inefficiently form the putative Arabidopsis IAA storage form IAA-
Leu (Barlier et al., 2000). The high-auxin phenotype in the yucca mutant is suppressed
by expressing iaaL (Zhao et al., 2001), a microbial IAA-conjugating enzyme (Glass and
Kosuge, 1986), suggesting that IAA-conjugating activities are insufficient in yucca. In
contrast, the trp2 and trp3 mutants apparently accumulate IAA conjugates but not free
IAA (Normanly et al., 1993) implying that the conjugation pathways are sufficient to
accommodate the accumulating IAA in this case.

A screen for mutants accumulating new conjugates following exposure to
prolonged high-IAA challenge revealed that the photorespiration mutant gluS
accumulates IAA-Gln at the expense of IAA-Asp following IAA treatment (Barratt et al.,
1999). Because the gluS mutant, which is defective in chloroplastic glutamate synthase,
has increased soluble Gln levels (Somerville and Ogren, 1980), this altered conjugate
profile implies that conjugates formed following IAA challenge are in part dependent on
amino acid pool sizes.

Remarkably, the enzymes that conjugate IAA to amino acids are encoded by
members of the GH3 family of auxin-induced genes (see “Auxin-Induced Transcripts”
section). These enzymes are in the luciferase superfamily (Staswick et al., 2002) and are
related to the JAR1 enzyme that conjugates the hormone jasmonic acid to amino acids
(Staswick and Tiryaki, 2004). Two members of the Arabidopsis GH3 family have been
uncovered as genes overexpressed in dwarf mutants with reduced apical dominance, *dfl1-D* (Nakazawa *et al.*, 2001) and *ydk1-D* (Takase *et al.*, 2004). *dfl1-D* is resistant to applied IAA, and both *dfl1-D* and *ydk1-D* have reduced lateral rooting and hypocotyl elongation (Nakazawa *et al.*, 2001; Takase *et al.*, 2004). These phenotypes are consistent with decreased free auxin levels, which would be expected when overexpressing an IAA-conjugating enzyme; indeed, IAA-Asp levels are elevated in *dfl1-D* (Staswick *et al.*, 2005). Further, disruption of certain GH3 genes confers hypersensitivity to specific forms of auxin conjugated by the encoded GH3 (Staswick *et al.*, 2005). The characterized GH3-like enzymes apparently prefer to synthesize inactivating (IAA-Asp and -Glu) over hydrolyzable (IAA-Ala and -Leu) conjugates *in vitro* (Staswick *et al.*, 2005); it will be interesting to learn which enzymes are responsible for synthesizing hydrolyzable conjugates *in vivo*.

In addition to IAA-amino acid conjugates, the esterified conjugate IAA-glucose is found in plants (Chisnell and Bandurski, 1988; Jakubowska and Kowalczyk, 2004; Tam *et al.*, 2000) and plant proteins with IAA glucosyltransferase activity have been identified (Jackson *et al.*, 2001; Jakubowska and Kowalczyk, 2004; Leznicki and Bandurski, 1988; Szerszen *et al.*, 1994). Maize *iaglu* (Szerszen *et al.*, 1994) and arabidopsis *UGT84B1A* (Jackson *et al.*, 2001) encode glucosyl transferases that conjugate IAA to glucose. Overexpressing *UGT84B1* renders plants resistant to exogenous IAA and disrupts gravitropism (Jackson *et al.*, 2002), consistent with a role in IAA inactivation. Paradoxically, free IAA levels are actually increased in *UGT84B1*-overexpressing lines (Jackson *et al.*, 2002). Because UGT84B1 also has considerable activity on IBA (Jackson *et al.*, 2001), it or a related glucosyl transferase may be responsible for the IBA-glucose formed from labeled IBA fed to arabidopsis seedlings (Ludwig-Müller and Epstein, 1993).

An enzyme that methylates the carboxyl side chain of IAA has recently been described (Zubieta *et al.*, 2003). This enzyme, IAMT1, is a member of a family of
carboxyl methyltransferases; other members methylate plant hormones such as jasmonic acid (JA) and salicylate (Zubieta et al., 2003). Methylation will increase the volatility of IAA, but it is not clear whether this modification activates or inactivates the hormone. In the case of JA, overexpressing the gene encoding jasmonate methyltransferase JMT increases resistance to a fungal pathogen (Seo et al., 2001), implying that methyl jasmonate is an active signaling molecule. Understanding of the in vivo roles of methylated IAA awaits analysis of plants with increased and decreased accumulation of this derivative.

Finally, as arabidopsis can convert IAA to IBA (Ludwig-Müller and Epstein, 1994), IBA synthase might contribute to IAA inactivation (Figure 1.3). An IBA synthase regulated by a variety of biotic and abiotic stresses (Ludwig-Müller, 2000) has been partially purified from maize (Ludwig-Müller and Hilgenberg, 1995), but the specific components or its effects on the free IAA pool have not been determined.

1.D. Auxin Transport

Although many tissues can synthesize auxin (Ljung et al., 2001), auxin transport is complex and highly regulated, involving many identified proteins. Chemical and genetic studies have revealed that transport of auxin to distant sites is clearly required for normal development. For example, IAA transport is necessary for proper lateral root development (Bhalerao et al., 2002; Reed et al., 1998b), vascular development (Mattsson et al., 1999), phyllotaxis (Reinhardt et al., 2003), embryonic axis development (Friml et al., 2003), and tropisms (Friml et al., 2002).

Auxin is produced largely in shoot apical regions, historically identified as the shoot apical meristem. However, application of auxin transport inhibitors blocks IAA accumulation in the shoot apex, suggesting that apical auxin is transported from other regions, probably young leaves and developing leaf primordia (Asvian-Kretchmer et al., 2002; Ljung et al., 2001). IAA is transported basipetally in shoots (Lomax et al., 1995)
and suppresses lateral shoot growth (Thimann, 1934). Both acropetal (Scott and Wilkins, 1968) and basipetal (Davies and Mitchell, 1972) transport occurs in roots. Opposing directions of auxin transport in roots is achieved by spatial separation, with acropetal transport in the central cylinder and basipetal transport in the epidermis (Tsurumi and Ohwaki, 1978).

Several arabidopsis mutants are defective in proteins mediating polar auxin transport. Among the earliest genes cloned that were defective in auxin-resistant mutants was AUX1, which encodes a transmembrane protein similar to amino acid permeases (Bennett et al., 1996). AUX1, and possibly other closely-related proteins, mediates influx of IAA into cells (Marchant et al., 1999). AUX1 is localized asymmetrically in the plasma membrane of certain cell files, facilitating directional auxin transport (Swarup et al., 2001; Swarup et al., 2004). Interestingly, IAA and the synthetic auxin 2,4-D appear to be AUX1 substrates, but aux1 remains sensitive to the synthetic compound NAA; indeed, NAA can restore aux1 gravitropism (Yamamoto and Yamamoto, 1998).

Once IAA has entered a cell via AUX1, several factors regulate efflux. The pin-formed (pin1) arabidopsis mutant is characterized by shoot meristem defects causing inflorescences to terminate in pin-shaped points generally lacking lateral organs (Okada et al., 1991). PIN1 is a member of a multigene family (including EIR1/AGRI/PIN2, another gene implicated in polar auxin transport) that encodes transmembrane auxin efflux facilitator proteins with homology to bacterial efflux carriers (Chen et al., 1998; Gälweiler et al., 1998; Luschnig et al., 1998; Müller et al., 1998a; Utsuno et al., 1998). Like AUX1, PIN1 is asymmetrically localized in the cell, consistent with a role in polar auxin efflux (Gälweiler et al., 1998).

The polar localization of PIN proteins is complex and dynamic. After a change in the gravity vector, PIN3 quickly moves from all lateral sides of columella cells specifically to the lateral side newly oriented toward gravity (Friml et al., 2002). Both PIN1 (Geldner et al., 2001) and PIN3 (Friml et al., 2002) rapidly cycle between the
plasma membrane and unidentified endosomal compartments. PIN1 cycling is inhibited
by the auxin transport inhibitor TIBA; movement from endosomal compartments to the
plasma membrane is likewise inhibited by the vesicular trafficking inhibitor BFA (Geldner
et al., 2001). PIN localization responds to cues from the PINOID serine-threonine
kinase; PINOID overexpression or disruption alters the polar localization of PIN proteins
in the cell (Friml et al., 2004). PIN cycling is actin-dependent (Geldner et al., 2001), and
links between actin, polar auxin transport, and gravitropism have been reported in several
plants (Hou et al., 2004; Hou et al., 2003; Sun et al., 2004).

Flavanoids, compounds that accumulate in specific locations in light-grown
arabidopsis (Buer and Munday, 2004), negatively regulate auxin transport (Brown et al.,
2001) and PIN1 cycling (Peer et al., 2004). PIN protein expression and localization are
altered in flavonoid mutants (Peer et al., 2004) and the arabidopsis transparent testa4
(tt4) mutant lacks flavonoids and has increased root basipetal and acropetal IAA transport
and delayed gravitropism (Buer and Munday, 2004).

In addition to PIN proteins, certain MULTIDRUG RESISTANCE-like (MDR)
proteins similar to mammalian MDR proteins are necessary for polar auxin transport in
arabidopsis (Noh et al., 2001), maize, and Sorghum bicolor (Multani et al., 2003). The
arabidopsis MDR proteins MDR1 and P-GLYCOPROTEIN 1 (PGP1) bind the auxin
transport inhibitor NPA (Noh et al., 2001). Basal PIN1 localization is disrupted in the
mdr1 pgp1 double mutant (Noh et al., 2003). PXA1, the peroxisomal membrane
transporter necessary for IBA β-oxidation (Zolman et al., 2001b), is also an MDR-like
protein.

The arabidopsis mutant transport inhibitor response 3 (tir3, allelic to doc1, big,
and umb1, see “Auxin Interactions with Other Hormones” section) has reduced auxin
transport (Ruegger et al., 1997). The mutant, resistant to root elongation inhibition by
NPA, has reduced NPA binding to microsomal fractions (Ruegger et al., 1997). Another
mutant with pleiotropic phenotypes, roots curl in NPA (rcn1, allelic to eer1, see “Auxin
Interactions with Other Hormones” section), is defective in a protein phosphatase 2A subunit (Deruère et al., 1999) and actually has increased basipetal auxin transport (Rashotte et al., 2001).

Differential transport of auxin precursors and storage forms offers a potential point of auxin regulation. Little is known about the transport of IAA conjugates. The auxl auxin influx carrier mutant (Bennett et al., 1996) is resistant to IAA-Ala and IAA-Leu (B Bartel, unpublished data), as well as to IAA (Pickett et al., 1990), so it is possible that at least these conjugates enter cells similarly to free IAA. Alternatively, the conjugate resistance of auxl could reflect failure to take up IAA freed by conjugate hydrolysis.

Similarly, the auxl mutant (Bennett et al., 1996) is less sensitive than wild type to root elongation inhibition by exogenous IBA (Zolman et al., 2000) and IAA competes effectively for labeled IBA uptake into arabidopsis seedlings (Ludwig-Müller et al., 1995), consistent with a shared importer. However, experiments with labeled IBA suggest that it is transported more efficiently than IAA in roots, and labeled IBA transport is unaffected in the auxl mutant (Rashotte et al., 2003). Thus, the IBA-resistance of auxl root elongation may reflect resistance to IAA derived from IBA.

IBA appears not to be a good substrate of the IAA efflux carrier. Unlike wild type, roots of the eir1/agr1/pin2 auxin transport mutant (Chen et al., 1998; Luschnig et al., 1998; Müller et al., 1998a; Utsuno et al., 1998) bend and enter vertically-oriented medium containing substrates of the efflux carrier, such as IAA or NAA (Utsuno et al., 1998). However, eir1 roots fail to bend and enter media containing 2,4-D (Utsuno et al., 1998) or IBA (Poupard and Waddell, 2000; Zolman et al., 2000), suggesting that IBA, like 2,4-D, does not use the IAA efflux carrier. In support of this observation, labeled IBA transport is unaffected in eir1 roots and is NPA-insensitive in wild-type roots (Rashotte et al., 2003). Proteins specifically mediating IBA transport remain to be identified.
1.E. Auxin Signaling

1.E.1. Auxin-induced transcripts

IAA biosynthesis, metabolism, and transport together ensure that appropriate auxin levels are in place to orchestrate plant development. How the signaling between auxin and downstream effectors occurs is the subject of much research. Auxin rapidly and transiently induces accumulation of at least three families of transcripts: SMALL AUXIN-UP RNAs (SAURs), GH3-related transcripts, and AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) family members.

SAUR transcripts accumulate rapidly after auxin exposure in soybean (Walker and Key, 1982) and many other species, including arabidopsis (Gil et al., 1994). Maize ZmSAUR2 is a small nuclear protein that, like the encoding transcript, is rapidly degraded (Knauss et al., 2003). The short half-lives of SAUR mRNAs appear to be conferred by downstream elements (DSTs) in the 3’ untranslated region of the messages (Sullivan and Green, 1996). Arabidopsis mutants that stabilize DST-containing RNAs, and thus stabilize SAUR transcripts, have no reported morphological phenotype (Johnson et al., 2000), and the function of these small RNAs remains unknown.

GH3 transcript accumulation is also induced by auxin (Hagen et al., 1984) and numerous plants have auxin-responsive GH3-like genes. At least some IAA-induced GH3 genes encode IAA-amino acid conjugating enzymes (Staswick et al., 2005), whereas several GH3-related proteins that are not auxin regulated function to adenylate or conjugate amino acids to molecules other than IAA, including jasmonic acid (Staswick and Tiryaki, 2004; Staswick et al., 2002). Thus, the auxin induction of GH3 genes likely serves to dampen the auxin signal by inactivating IAA via conjugation (see “IAA Inactivation” section).

Like the GH3 family, Aux/IAA transcripts accumulate following auxin exposure and the encoded proteins also apparently serve to dampen auxin signaling. Induction of
some Aux/IAA genes occurs within minutes of auxin application and does not require new protein synthesis (Abel et al., 1994; Abel and Theologis, 1996). The Aux/IAA family includes 28 proteins in arabidopsis (Liscum and Reed, 2002; Remington et al., 2004), and homologous genes, some of which are also auxin-induced, are present in other plants. The encoded proteins share extensive sequence identity in four conserved domains. Domain I is a transcriptional repressor (Tiwari et al., 2004). Domain II is critical for Aux/IAA instability; several mutations in this domain have been isolated as gain-of-function alleles that stabilize the proteins and confer auxin-resistant phenotypes. In fact, a 13-amino acid fragment of domain II from IAA7/AXR2 is sufficient to confer auxin-mediated degradation to a fusion protein (Ramos et al., 2001). Domains III and IV are involved in homodimerization and heterodimerization with other Aux/IAA proteins and with AUXIN REPONSE FACTORs (ARFs; Hardtke et al., 2004; Kim et al., 1997; Ulmasov et al., 1999b).

Many genes with auxin-induced expression, including most SAUR, GH3, and Aux/IAA genes, share a common sequence in their upstream regulatory regions, TGTCTC or variants, first identified from the promoter region of the pea PS-IAA4/5 gene (Ballas et al., 1993). Regions including sequence, known as the Auxin-Responsive Element, or AuxRE, confer auxin-induced gene expression in synthetic constructs (Ulamasov et al., 1995; Ulmasov et al., 1997b). More recently, genome-wide profiling experiments have revealed a wealth of auxin-induced genes (Cluis et al., 2004; Himanen et al., 2004; Pufky et al., 2003; Sawa et al., 2002), many of which contain AuxREs in putative regulatory regions (Nemhauser et al., 2004; Pufky et al., 2003).

1.E.2. Auxin Response Factors mediate auxin-induced changes in gene expression

Identification of the AuxRE led to isolation of ARF1, the founding member of the AuxRE-binding protein family, using a yeast one-hybrid screen (Ulamasov et al., 1997a). ARF proteins can either activate or repress target gene transcription, depending on the
nature of a central domain (Tiwari et al., 2003; Ulmasov et al., 1999a). ARFs can bind tandem repeat AuxRE sequences as homodimers, dimers with other ARFs, or dimers with repressive Aux/IAA proteins (Ulmasov et al., 1997a; Ulmasov et al., 1999a). Auxin responsiveness depends on ARF motifs similar to Aux/IAA protein domains III and IV and is mediated through dimerization with Aux/IAA proteins (Tiwari et al., 2003). Because the dimerization domains are dispensable for ARF activation of reporter genes in Daucus carota protoplast assays, it is likely that ARFs can function as monomers (Tiwari et al., 2003).

Mutations in several arabidopsis ARF genes confer gene-specific developmental defects. Mutations in ETTIN/ARF3 (ETT) lead to floral abnormalities (Sessions et al., 1997; Sessions and Zambryski, 1995) that can be phenocopied by NPA application to flowers (Nemhauser et al., 2000). Genetic interaction between ETT and the SEUSS gene is necessary for proper floral development (Pfluger and Zambryski, 2004). Further, seuss mutants are defective not only in flower morphology, but are generally defective in auxin responses (Pfluger and Zambryski, 2004).

A screen for suppressors of a hookless1 (hls1) mutant, which fails to form an ethylene-mediated apical hook in darkness (Lehman et al., 1996), identified hookless1 suppressor 1/arf2 (Li et al., 2004). Ethylene acts through HLS1 to negatively regulate ARF2 levels and achieve an apical hook; ARF2 overexpression, like HLS1 deficiency, inhibits apical hook formation (Lehman et al., 1996; Li et al., 2004). Loss of arf2 function has no observable effect on apical hook formation when not in combination with hls1, but does cause various defects in adult morphology (Lehman et al., 1996; Li et al., 2004).

Defects in MONOPTEROS/ARF5 (MP), a transcriptionally-activating ARF, result in aberrant seedling morphology, often with a single cotyledon and a loss of basal structures (Hardtke and Berleth, 1998). Mutations in a second activating ARF, NON-PHOTOTROPIC HYPOCOTYL4 (NPH4/TIR5/MSG1/ARF7), confer deficient shoot
phototropism, an auxin-mediated process (Harper et al., 2000). While the phenotypes of mp/arf5 and nph4/arf7 initially appeared unrelated, combining a weak allele of mp with nph4 in a double mutant enhances the mp fused cotyledon phenotype (Hardtke et al., 2004), suggesting some functional overlap between the two ARFs.

Thus, mutation of several ARF genes confers developmental phenotypes, and abundant evidence from studies employing reporter constructs in protoplast assays implicates ARFs in auxin responsive transcription. Studies in intact plants are beginning to directly link ARFs with auxin-responsive transcription at native promoters. For example, nph4/arf7 mutant seedlings display dramatically reduced levels of several auxin induced transcripts both before and after auxin application (Stowe-Evans et al., 1998) and expression of several Aux/IAA genes responds to MP/ARF5 levels (Mattsson et al., 2003).

The diversity of arf mutant phenotypes makes it clear that the rules governing the interactions between ARFs and AuxREs will be complex; the fact that only a few arf mutants have been reported indicates that much of this complexity remains to be uncovered. ARF8 disruption leads to mild but significant high-auxin phenotypes such as stronger apical dominance, increased lateral root number, and a longer hypocotyl in the light; conversely, ARF8 overexpression results in opposite phenotypes, suggesting reduced auxin response (Tian et al., 2004). These observations are initially counterintuitive, because ARF8 is an activating ARF (Ulmasov et al., 1999a). However, these results can be neatly explained by the observation that ARF8 induces expression of several GH3 genes (Tian et al., 2004) involved in auxin inactivation (Staswick et al., 2005). Indeed, free IAA levels are reduced in hypocotyls of ARF8 overexpression lines (Tian et al., 2004), demonstrating the intimate connection between auxin responses and auxin levels.
1.E.3. Aux/IAA proteins repress ARF function

Aux/IAA proteins interact with ARF proteins via C-terminal domains III and IV conserved between the Aux/IAA family and most ARF proteins (Ulmasov et al., 1999b). At least some Aux/IAA proteins can directly repress transcription (Tiwari et al., 2004), and the interaction of Aux/IAA proteins with activating ARF proteins can prevent transcriptional activation in protoplast assays (Tiwari et al., 2003).

It may seem counterintuitive that expression of some Aux/IAA genes, which function to repress auxin signaling, is auxin-induced. However, Aux/IAA protein levels plummet immediately following auxin exposure (see below). Increased transcription of Aux/IAA genes after an auxin stimulus is likely mediated by ARF proteins via AuxREs in Aux/IAA promoter regions. Thus, Aux/IAA accumulation is subject to negative feedback; Aux/IAA protein levels decline after auxin exposure, allowing increased transcription of Aux/IAA genes and thereby ensuring a transient auxin response.

Gain-of-function Aux/IAA mutations generally reduce auxin sensitivity in root elongation assays and confer dramatic auxin-related developmental defects, including altered gravitropism and apical dominance in axr2/iaa7 (Nagpal et al., 2000; Wilson et al., 1990), axr3/iaa17 (Rouse et al., 1998), and axr5/iaa1 (Yang et al., 2004), severe lateral root defects in iaa28 (Rogg et al., 2001) and slr/iaa14 (Fukaki et al., 2002), photomorphogenic defects in shy2/iaa3 (Soh et al., 1999; Tian and Reed, 1999), hypocotyl tropism defects in msg2/iaa19 (Tatematsu et al., 2004), and embryonic patterning defects in bdl/iaa12 (Hamann et al., 2002). Remarkably, these dominant missense mutations all map to a small region of domain II and several have been shown to stabilize the encoded Aux/IAA proteins (Gray et al., 2001; Ramos et al., 2001; Tiwari et al., 2001; Worley et al., 2000; Zenser et al., 2001), underscoring the importance of this region in vivo. In contrast to the dramatic defects conferred by stabilizing Aux/IAA proteins, the few reported loss-of-function Aux/IAA alleles confer only subtle phenotypes. Most were isolated as suppressors of gain-of-function alleles mutated in the same gene.
(Nagpal et al., 2000; Rouse et al., 1998; Tian and Reed, 1999). Loss-of-function shy2/iaa3 mutants have large cotyledons and short hypocotyls (Tian and Reed, 1999), and null axr2/iaa7 mutants have slightly longer hypocotyls than wild type (Nagpal et al., 2000). The dramatic phenotypes of the gain-of-function Aux/IAA mutants coupled with the subtle effects of losing individual Aux/IAA genes suggests that these genes play important but largely overlapping roles in wild-type plants.

1.E.4. Aux/IAA degradation is mediated by the SCF<sup>TIR1</sup> ubiquitin ligase

The Aux/IAA proteins, which inhibit auxin responses, are unstable even in the absence of a stimulus (Abel et al., 1994). Auxin application further destabilizes Aux/IAA proteins (Gray et al., 2001; Zenser et al., 2001), which is presumed to free activating ARF proteins from repression and thereby allow auxin-induced gene expression (Figure 1.4). The transient nature of auxin-induced transcription is likely to result in part from many of the Aux/IAA genes themselves being transcriptionally induced by auxin (Abel et al., 1995b).

Aux/IAA proteins are unstable because they are targets of ubiquitin-mediated degradation. Ubiquitin is covalently attached to substrate proteins in a three-step process that begins with activation of the ubiquitin C-terminus by an E1 enzyme, followed by ubiquitin transfer from the E1 to an E2 intermediary protein. Finally, the E2-ubiquitin complex is brought into proximity to the target protein via a specificity-providing E3 protein or protein complex, which mediates the ubiquitination of target proteins. Once targets are multiply ubiquitinated, they are substrates for degradation by the 26S proteasome. One type of E3 complex is the Skp1-Cullin-F-box (SCF) class, named for the defining components. SCF complexes are comprised of mostly general subunits, while one of numerous F-box proteins confers target specificity. The F-box protein associates with a cullin (CUL1) via a SKP adapter protein named ASK, for *arabidopsis* SKP1-like. CUL1 also binds to the ubiquitin E2-interacting protein RBX1 (also known
Figure 1.4. Auxin signaling.
TIR1 complex for ubiquitination (Ub) and subsequent destruction by the 26S proteasome. The activating ARF, with a Gln-rich (Q) middle domain, is then freed to promote auxin-induced gene expression. The ubiquitin-related protein RUB is activated by ECR1/AXR1, transferred to RCE1, and conjugated to CUL1 to regulate SCF activity. RUB is cleaved from CUL1 by a subunit of the COP9 signalosome, a protein complex that may function as a proteasome lid.
as HRT1 or ROC1). Thus, the SCF complex recruits an E2 protein bearing ubiquitin to a specific protein target to facilitate ubiquitination of the target protein.

Several auxin-resistant mutants stabilize Aux/IAA proteins by disrupting components of the SCF<sup>TIR1</sup> ubiquitin ligase or proteins that regulate the SCF (Table 1.4). <i>tir1</i> mutants are resistant to auxins, auxin transport inhibitors, and the auxin mimic sirtinol (Ruegger et al., 1998; Zhao et al., 2003b). <i>TIR1</i> encodes a leucine-rich repeat-containing F-box protein (Ruegger et al., 1998) that lends Aux/IAA ubiquitination specificity to the SCF<sup>TIR1</sup> complex. The auxin-resistant <i>axr6</i> mutant is defective in CUL1 (Hellmann et al., 2003), and misexpressing RBX1 results in auxin resistance (Gray et al., 2002). Also, ASK1 is necessary for proper auxin response, and ASK1 and ASK2, members of a 19-member family (Farrás et al., 2001), interact with the TIR1 F-box (Gray et al., 1999).

SCF<sup>TIR1</sup> regulation is complex, and auxin resistance mutant screens have revealed regulatory components as well. <i>AXRI</i> encodes one subunit of a ubiquitin E1-like enzyme complex (Leyser et al., 1993) with the partner protein E1-LIKE CONJUGATING ENZYME-RELATED 1 (ECR1; del Pozo et al., 2002; del Pozo et al., 1998). The AXR1-ECR1 dimer activates and transfers RELATED TO UBIQUITIN 1 (RUB1, known as NEDD8 in animals) to the RUB1-CONJUGATING ENZYME RCE1 (Dharmasiri et al., 2003b). RUB is subsequently transferred to a specific lysine on CUL1 (del Pozo and Estelle, 1999), apparently using RBX1 as the RUB E3 ligase to facilitate RUB transfer from RCE1 to CUL1 (Gray et al., 2002). Although mutants defective in <i>ECR1</i> have not been reported, overexpression of ECR1 with the active site cysteine mutated confers developmental defects in the shoot (del Pozo et al., 2002). Defects in AXR1 (Estelle and Somerville, 1987; Lincoln et al., 1990), RUB (Bostick et al., 2004), or RCE1 (Dharmasiri et al., 2003b; Larsen and Cancel, 2004) confer auxin resistance,
<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Loss-of-function phenotype*</th>
<th>Assay</th>
</tr>
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</table>
| **TIR1** | **auxin F-box**   | auxin resistant\(^1\)  
NPA, CPD resistant\(^1\)  
reduced lateral root number\(^1\)  
hypocotyl elongation defect\(^1\)  
enhances axr1-12 dwarfism\(^2\)  
enhances cand1 dwarfism\(^2\) | root elongation inhibition  
root elongation inhibition  
growth at elevated temperature |
| **CUL1/AXR6** | SCF scaffold | auxin resistant\(^3\)  
embryo lethal (null)\(^4\) | root elongation inhibition |
| **ASK** | **CUL1/F-box adapter** | auxin resistant \(ask1\)\(^5\)  
reduced lateral root number \(ask1\)\(^5\)  
dwarf \(ask1\)\(^5\)  
floral abnormalities \(ask1\) and \(ask1\)\(^6\)  
embryo lethal \(ask1 ask2\)\(^6\) | root elongation inhibition |
| **RBX1** | **CUL1/E2 adapter** | auxin resistant\(^7\)  
reduced lateral root number\(^7\)  
dwarfism\(^1\)  
MeJA resistant\(^7\)  
delayed cold-induced gene expression\(^7\) | root elongation inhibition  
root elongation inhibition  
northern blot |
| **RUB1 RUB2** | ubiquitin-like modifier | auxin resistant\(^8\)  
reduced lateral root number\(^8\)  
dwarfism\(^8\)  
ethylene overproduction\(^8\)  
embryo lethal (null)\(^8\) | root elongation inhibition  
hypocotyl elongation in darkness, GC |
| **AXR1** | **RUB1 activating enzyme component** | auxin resistant\(^9\)  
reduced gravitropism\(^10\)  
dwarfism (severe alleles)\(^9\)  
MeJA resistant\(^11\)  
ACC resistant\(^12, 13\)  
enhances cop10-4 deetiolation\(^7\)  
floral abnormalities\(^14\)  
delayed cold-induced gene expression\(^7\) | root elongation inhibition  
root reorientation  
root elongation inhibition  
hypocotyl elongation inhibition in darkness  
growth in darkness  
northern blot |
| **ECR1** | **RUB activating enzyme component** | reduced auxin-induced gene expression\(^14\)  
dwarfism\(^14\)  
floral abnormalities\(^14\) | northern blot of wild-type plants  
transformed with a mutant version of ECR1 |

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\(^1\) From DiFiglia et al. (2007)  
\(^2\) From Ben-Asher et al. (2007)  
\(^3\) From Nakashima et al. (2005)  
\(^4\) From Gómez et al. (2007)  
\(^5\) From Xu et al. (2007)  
\(^6\) From Zhao et al. (2008)  
\(^7\) From Chen et al. (2009)  
\(^8\) From Hu et al. (2009)  
\(^9\) From Tone et al. (2009)  
\(^10\) From Lu et al. (2009)  
\(^11\) From Lee et al. (2010)  
\(^12\) From Zhang et al. (2010)  
\(^13\) From Li et al. (2011)  
\(^14\) From Wang et al. (2011)
Table 1.4. Auxin-related SCF components and SCF-regulatory arabidopsis genes continued.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Loss-of-function phenotype*</th>
<th>Assay</th>
</tr>
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<tbody>
<tr>
<td>RCE1</td>
<td>RUB E2 enzyme</td>
<td>auxin resistance(^{10}), reduced lateral root proliferation(^{10}), reduced gravitropism(^{10}), dwarfism(^{10}), MeJA resistance(^{10}), ethylene overproduction(^{15}), reduced hypocotyl elongation in darkness(^{15})</td>
<td>root elongation inhibition, lateral root induction by auxin, root reorientation, root elongation inhibition, gas chromatography</td>
</tr>
<tr>
<td>CSN5</td>
<td>COP9 signalosome component</td>
<td>auxin resistance(^{7}), reduced lateral root number(^{7}), dwarfism(^{7}), MeJA resistance(^{7}), delayed cold-induced gene expression(^{7})</td>
<td>root elongation inhibition, root elongation inhibition, northern blot</td>
</tr>
<tr>
<td>CAND1/ETA2</td>
<td>SCF regulator</td>
<td>auxin resistance(^{2,13,17}), reduced lateral root number(^{2}), dwarfism(^{2}), MeJA resistance(^{17}), ACC resistance(^{6}), reduced apical hook(^{13}), floral abnormalities(^{17}), ABA resistance(^{13}), enhanced red light response(^{13})</td>
<td>root elongation inhibition, root elongation inhibition, hypocotyl elongation inhibition, growth in darkness, root elongation inhibition, hypocotyl elongation inhibition</td>
</tr>
<tr>
<td>SGT1b</td>
<td>SCF regulator</td>
<td>auxin resistance(^{16}), reduced lateral root number(^{16}), MeJA resistance(^{16})</td>
<td>root elongation inhibition, root elongation inhibition</td>
</tr>
</tbody>
</table>

*Red, auxin-related phenotypes; blue, ethylene-related phenotypes; purple, jasmonate-related phenotypes; green, floral development phenotypes.

\(^{1}\) (Ruegger et al., 1998); \(^{2}\) (Chuang et al., 2004); \(^{3}\) (Hellmann et al., 2003); \(^{4}\) (Shen et al., 2002); \(^{5}\) (Gray et al., 1999); \(^{6}\) (Zhao et al., 2003a); \(^{7}\) (Schwechheimer et al., 2002); \(^{8}\) (Bostick et al., 2004); \(^{9}\) (Lincoln et al., 1990); \(^{10}\) (Dharmasiri et al., 2003b); \(^{11}\) (Tiryaki and Staswick, 2002); \(^{12}\) (Xu et al., 2002); \(^{13}\) (Cheng et al., 2004); \(^{14}\) (del Pozo et al., 2002); \(^{15}\) (Larsen and Cancel, 2004); \(^{16}\) (Gray et al., 2003) 17(Feng et al., 2004).
and *axr1 rce1* double mutants have embryonic defects similar to *mp/arf5* (Dharmasiri *et al.*, 2003b), prompting the hypothesis that RUB modification positively regulates SCF^{TRI} activity.

Recent results suggest a more complicated situation. **CONSTITUTIVE PHOTOMORPHOGENESIS 9** (COP9) is a component of the COP9 signalosome complex, which resembles the 26S proteasome lid (Li and Deng, 2003). COP9 signalosome activity is necessary both for RUB removal from CUL1 and for proper auxin response (Schwechheimer *et al.*, 2001). In addition, overexpressing *RBX1* results in increased RUB-CUL1 levels and 2,4-D resistance (Gray *et al.*, 2002). Thus, it appears that RUB addition to and removal from CUL1 are both required for SCF^{TRI} function, implying a necessary RUB modification cycle. However, RUB deconjugation is separable from at least some developmental roles of the COP9 signalosome (Wang *et al.*, 2002). Evidence that the COP9 signalosome interacts with proteasome components indicates that the COP9 signalosome may be a *bona fide* proteasome lid (Li and Deng, 2003; Peng *et al.*, 2003; Schwechheimer and Deng, 2001) that could function directly in both RUB removal and ubiquitin-mediated substrate degradation.

A screen for mutations that enhance the 2,4-D resistance of *tir1* uncovered several previously-identified SCF^{TRI} components as well as *enhancer of tir1-1 auxin resistance 3 (eta3; Gray et al., 2003).* The *eta3* single mutant phenotype is similar to *tir1*, and when combined in *tir1 eta3*, enhances the *tir1* lateral root defect, short hypocotyl, and 2,4-D resistance phenotypes (Gray *et al.*, 2003). *eta3* is deficient in SGT1b (Gray *et al.*, 2003), a homolog of yeast SGT1, which interacts with SCF complexes (Kitagawa *et al.*, 1999). While the role of SGT1b remains obscure, *eta3* is not deficient in SCF^{TRI} assembly or auxin-responsive interaction of TIR1 with an Aux/IAA protein *in vitro* (Gray *et al.*, 2003).

Further modulation of SCF is achieved by a plant ortholog of the human Cullin-Associated and Neddylation-Dissociated (CAND) protein, which is predicted to regulate
SCF assembly (Liu et al., 2002; Oshikawa et al., 2003; Zheng et al., 2002). Defects in arabidopsis CAND1/ETA2 result in auxin resistance (Cheng et al., 2004; Chuang et al., 2004; Feng et al., 2004). Though CUL1 and RUB-CUL1 levels appear wild type in cand1 mutants (Chuang et al., 2004; Feng et al., 2004), pulldowns with the SCF<TIR1>-interacting domain of AXR2/IAA7 reveal increased CUL1, but decreased ASK1, association (Chuang et al., 2004). Further experiments examining SCF component and substrate interactions in cand1 are needed to understand these results, but it is clear that CAND1 is essential for proper SCF<TIR1> function.

1.E.5. Auxin induction of Aux/IAA-TIR1 interaction

Auxin application to plants (Gray et al., 2001) or addition to plant cell lysate (Dharmasiri et al., 2003a) can quickly induce Aux/IAA domain II interaction with TIR1. Thus, auxin could destabilize Aux/IAA proteins in planta by promoting association with the SCF<TIR1> E3 ubiquitin ligase complex. The in vitro dissection of this TIR1-Aux/IAA interaction holds promise to reveal at least one of the long-sought auxin receptors.

Many substrates of SCF complexes require modification prior to ubiquitination (Deshaies, 1999). Several observations led to the early hypothesis that alterations to proline residues in domain II could provide a mechanism for auxin promotion of TIR1-Aux/IAA domain II interaction. First, several dominant auxin-resistant mutants harbor mutations altering one of two adjacent prolines within domain II (Fukaki et al., 2002; Hamann et al., 2002; Nagpal et al., 2000; Rogg et al., 2001; Rouse et al., 1998; Soh et al., 1999; Tatematsu et al., 2004; Tian and Reed, 1999; Yang et al., 2004); many of these mutations are known to stabilize the mutant Aux/IAA protein (Ouellet et al., 2001; Worley et al., 2000). In addition, a mutant resistant to the auxin-like effects of sirtinol is defective in SIR1, a protein containing a domain present in certain Peptidyl Prolyl cis-trans Isomerases (PPIases; Zhao et al., 2003b), and the parvulin-class PPIase inhibitor juglone inhibits Aux/IAA-TIR1 interaction (Dharmasiri et al., 2003a; Tian et al., 2003).
However, although proline modification in Aux/IAA domain II may be relevant to TIR1 interaction, it is not the change induced by auxin. Mass spectroscopic analysis of AXR3/IAA17 domain II reveals possible proline hydroxylation, but this state is not affected by auxin addition (Kepinski and Leyser, 2004). Indeed, auxin does not appear to cause any covalent modification of domain II (Kepinski and Leyser, 2004). In addition, sirtinol does not promote Aux/IAA-TIR1 interaction (Kepinski and Leyser, 2004); therefore, sirtinol (and SIR1) influence Aux/IAA turnover in some other way, perhaps by regulating SCF^{TIR1} or proteasome activity. SIR1 is in fact more closely related to E1 ligases than to PPIases, suggesting yet another ubiquitin-like modification (in addition to the known roles of ubiquitin and RUB) important for auxin responsiveness. Finally, juglone affects TIR1 or an associated protein, not Aux/IAA domain II, to repress Aux/IAA-TIR1 association (Kepinski and Leyser, 2004).

The signal by which auxin promotes Aux/IAA-TIR1 interaction remains unclear. Tantalizing results show that *in vitro* treatment of extracts with NAA induces modification of TIR1 or a TIR1-associated protein rather than the Aux/IAA proteins (Kepinski and Leyser, 2004). Thus the most direct hypothesis, that TIR1 is itself an auxin receptor and that association of auxin with TIR1 promotes its binding and ubiquitination of substrate Aux/IAA proteins, will soon be testable.

1.F. Auxin Interactions With Other Hormones

1.F.1. Auxin modulates hormone levels

Physiological studies suggest many links between phytohormones. Several other hormones modulate or are modulated by auxin levels and responses. One of the most-told stories in plant biology is the relationship between auxin and cytokinin, which can be employed *in vitro* to induce root and shoot development, respectively (Skoog and Miller, 1957). Auxin and cytokinin levels are inversely correlated *in vivo* (Eklöf *et al.*., 2000) and auxin treatment can rapidly inhibit cytokinin biosynthesis (Nordström *et al.*., 2004).
Auxin and the gaseous hormone ethylene are also intimately linked. Exogenous auxin exposure stimulates ethylene production (Morgan and Hall, 1962) through induction of a gene encoding the rate-limiting step in ethylene biosynthesis (Abel et al., 1995a). Conversely, ethylene inhibits lateral (Burg and Burg, 1966) and basipetal (Suttle, 1988) auxin transport.

As with ethylene, auxin elicits increased gibberellic acid (GA) production (Ross et al., 2000), and basipetally-transported auxin is necessary for the production of the active gibberellins GA$_1$ and GA$_3$ in barley (Wolbang et al., 2004). GAs act, at least in part, through promoting degradation of DELLA repressors (Silverstone et al., 2001); disrupting auxin transport precludes GA-mediated DELLA protein degradation (Fu and Harberd, 2003).

Auxin response is also connected to brassinosteroids (BRs), which act in concert with auxin to promote root gravitropic curvature in maize (Kim et al., 2000). BR and auxin treatments induce accumulation of many of the same transcripts (Goda et al., 2004; Nemhauser et al., 2004). Exposure to the hormone abscisic acid (ABA) decreases free IAA levels while increasing esterified IAA conjugates in muskmelon ovaries (Dunlap and Robacker, 1990). Antagonistic to auxin, exogenous ABA inhibits lateral root formation (De Smet et al., 2003).

1.F.2. The SCF links auxin signaling with other stimuli

Several mutants with defects in auxin response are also defective in responses to other phytohormones, suggesting communication or cross-talk between phytohormone response pathways. For example, mutations in the general components comprising or regulating the SCF cause deficient responses to multiple hormonal and environmental stimuli (Schwechheimer et al., 2002). Though the full phenotypic complexity of mutants compromised in multiple pathways is likely to be revealed in years to come, pleiotropic
defects have already been reported for many mutants with compromised auxin response (Table 1.4).

axr1 mutants fail to efficiently modify CUL1 with RUB (del Pozo et al., 2002) and are resistant to the root elongation inhibition caused by auxins (Estelle and Somerville, 1987), cytokinin, ethylene (Timpte et al., 1995), methyl jasmonate (Schwechheimer et al., 2002), and epi-brassinolide (Tiryaki and Staswick, 2002). These data suggest that RUB modification is necessary for active SCF complexes and hormone responses in addition to SCF

Indeed, SCFCOI1 is necessary for jasmonate responses (Xie et al., 1998), and the RUB E2 enzyme-defective mutant rcel1 is auxin and methyl-jasmonate resistant (Dharmasiri et al., 2003b) and overproduces ethylene (Larsen and Cancel, 2004). In addition, reduced expression of the CSN5 subunit of the COP9 signalosome that removes RUB from CUL1, or of the SCF component RBX1 that adds RUB to CUL1, or defects in the putative CUL1-regulatory protein SGT1b, each cause deficient auxin and methyl-jasmonate responses (Gray et al., 2003; Schwechheimer et al., 2001).

axr1 and cand1 are also resistant to hypocotyl elongation inhibition by the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) in darkness (Cheng et al., 2004). Apical hook formation in darkness, an auxin-mediated differential growth response promoted by ethylene, is reduced in cand1 (Chuang et al., 2004). However, the RUB1 E2 conjugating enzyme mutant rcel1, though defective in auxin response (Dharmasiri et al., 2003b), overproduces ethylene and has an exaggerated triple response (Larsen and Cancel, 2004). Likewise, reducing RUB levels causes ethylene overproduction and enhanced triple response (Bostick et al., 2004). Indeed, SCF complexes with the F-box proteins EBF1 and EBF2 repress ethylene response by targeting the ethylene response transcriptional activator EIN3 for degradation; ebf1 ebf2 mutants show constitutive ethylene responses (Gagne et al., 2004; Guo and Ecker, 2003; Potuschak et al., 2003).
Gibberellins promote degradation of repressor DELLA proteins mediated by SCF$^{SLY1}$ (Dill et al., 2004; McGinnis et al., 2003) and the functionally-redundant SCF$^{SNE}$ (Strader et al., 2004). Though no altered morphological response to GA has been reported in general SCF mutants, a DELLA protein-GFP fusion is more stable in axr1-12 than wild type following GA application (Fu and Harberd, 2003).

In addition to hormone responses, SCF complexes can modulate development and environmental responses. For example, the F-box protein UFO is necessary for floral development (Samach et al., 1999). axr1 plants and transgenic plants overexpressing an inactive ECR1 both bear abnormal flowers; introducing the inactive ECR1 transgene into axr1 enhances the severity of axr1 floral defects (del Pozo et al., 2002). Also, though a role for an F-box complex in cold response has not been demonstrated, axr1, csn5, and rbx1 each display delayed cold-regulated gene induction (Schwechheimer et al., 2002).

The full impact of many response pathways feeding through a single type of protein degradation apparatus, the SCF, remains to be determined. However, it is tempting to imagine competition for the shared SCF components among hundreds of specificity-determining F-box proteins. Additionally, it is possible that regulatory mechanisms such as RUB modification may differentially regulate specific SCF complexes. Further, different phytohormones modulate SCF activity in opposing ways: repressive proteins are degraded in response to auxin (Rogg and Bartel, 2001), gibberellin (Itoh et al., 2003), and presumably jasmonate (Xu et al., 2002), whereas ethylene impedes destruction of the transcriptional activator EIN3 by SCF$^{EBF1/EBF2}$ (Gagne et al., 2004; Guo and Ecker, 2003; Potuschak et al., 2003).

1. F.3. Additional genes bridge auxin response with other stimuli

In addition to factors affecting SCF function, defects in several proteins with less-defined roles in auxin response display altered response to other hormones and environmental conditions. For example, several lines of evidence suggest that protein
phosphorylation influences response to various stimuli including auxin exposure. First, auxin, salicylic acid, wounding, and salt treatments induce MAPK activity in Arabidopsis roots, whereas kinase inhibitors block auxin inducibility of a reporter gene (Mockaitis and Howell, 2000).

Second, expression of constitutively-active forms of the ANP family members of Arabidopsis mitogen activated protein kinase kinase kinases (MAPKKKs) dampens responsiveness of an auxin-inducible reporter gene construct in protoplasts (Kovtun et al., 2000). The same kinase family positively regulates responses to oxidative stress, and, like constitutively-active ANPs, \( \text{H}_2\text{O}_2 \) exposure inhibits auxin-responsive reporter gene induction (Kovtun et al., 2000). Likewise, a constitutively-active form of the related tobacco MAPKKK NPK1 dampens auxin responsiveness of a reporter gene (Kovtun et al., 1998) and confers resistance to freezing, heat, and salt stresses (Kovtun et al., 2000). Based on these results and auxin activation of kinase activity, there is both a positive and negative modulation of auxin responsiveness by kinases.

Third, a mutant isolated because of altered response to an auxin transport inhibitor, roots curl in NPA 1 (rcn1) is deficient in a protein phosphatase 2A subunit (Deruère et al., 1999; Garbers et al., 1996). In addition to having increased IAA transport (Rashotte et al., 2001) and overproducing ethylene (Larsen and Cancel, 2003; Larsen and Chang, 2001), rcn1 is defective in NPA, gravity (Rashotte et al., 2001), and ABA (Kwak et al., 2002) responses. Likewise, protein phosphatase inhibitors phenocopy rcn1 gravitropism and IAA transport defects when applied to wild-type Arabidopsis (Rashotte et al., 2001).

Finally, the indole-3-butyric acid-response mutant ibr5 is deficient in an apparent dual-specificity phosphatase (Monroe-Augustus et al., 2003). ibr5 is resistant to auxins and ABA (Monroe-Augustus et al., 2003). In addition, ibr5 makes fewer lateral roots, a longer primary root, and a shorter hypocotyl than wild type, phenotypes consistent with generally deficient auxin response (Monroe-Augustus et al., 2003).
In addition to phosphorylation-related genes, genes classically involved in auxin signaling are involved in response to other environmental cues. For example, Aux/IAA and ARF proteins are involved in light responses. Stabilizing gain-of-function mutations in shy2/iaa3, axr2/iaa7, and axr3/iaa17 cause de-etiolation in darkness (Kim et al., 1996; Nagpal et al., 2000; Reed et al., 1998a). Moreover, the constitutive photomorphogenic mutant long hypocotyl 5 (hy5; Koornneef et al., 1980) can be partially rescued by overexpressing AXR2/IAA7 (Cluis et al., 2004). Further, hookless1 (hls1) fails to make an apical hook in darkness (Lehman et al., 1996), and this phenotype is suppressed by mutation of the auxin response factor ARF2 (Li et al., 2004).

The arabidopsis mutant abscisic acid insensitive 3 (abi3) was isolated because of ABA-insensitive germination and is deficient in a transcription factor (Giraudat et al., 1992). In addition to ABA resistance, abi3 is resistant to lateral root proliferation induced by auxin and lateral root repression by the auxin transport inhibitor NPA, and ABI3::GUS reporter accumulation is induced by auxin exposure (Brady et al., 2003). Additionally, certain mutants defective in auxin transport exhibit altered responses to other stimuli. The auxin influx mediator aux1 is resistant to specific auxins and to ethylene (Pickett et al., 1990). rcn1 has increased auxin transport and numerous developmental and phytohormone phenotypes (see above). Further, mutations in the auxin transport facilitator TRANSPORT INHIBITOR RESPONSE 3 (TIR3; Ruegger et al., 1997) cause not only low-auxin phenotypes, but also altered responses to light, ethylene, cytokinin, and gibberellin (Kanyuka et al., 2003). tir3 alleles have been isolated as dark overexpression of CAB1 (doc1; Li et al., 1994), the cytokinin-resistant mutant umbrella1, and attenuated shade avoidance1 (Kanyuka et al., 2003); the gene has been renamed BIG (Gil et al., 2001).
Chapter 2: Materials and Methods

2.A. Plant materials and growth conditions

2.A.1. Arabidopsis

All mutants that are subjects of this thesis are in Arabidopsis thaliana Columbia-0 (Col-0) accession. Mutants were crossed to accessions Landsberg erecta (Ler) or Wassilewskija (Ws) for mapping. Seeds were sterilized by incubation in 20% [v/v] household bleach 0.01% [v/v] TRITON X-100 detergent solution for 12 m., followed by two washes in sterile water and suspension in sterile 0.1% [w/v] agar (Last and Fink, 1988). Plants were grown in sterile conditions on plant nutrient medium (PN; Haughn and Somerville, 1986) supplemented with 0.5% [w/v] sucrose (PNS) unless indicated. Plates were sealed with Leukopor surgical tape (LekTek Corp., Minnetonka, MN) to prevent contamination but allow gas exchange. Seedlings were grown in Percival incubators under continuous illumination (25-45 μE m⁻² s⁻¹) at 22 °C unless indicated. Some plants were then transferred to soil (MetroMix 200, Scotts, Marysville, OH) and grown to maturity under continuous white light (Sylvania Cool White fluorescent bulbs, Danvers, MA) at 22-25 °C.

2.A.2. Bean, pea, and corn

Seeds for bean (Phaseolus vulgaris) Blue Lake Bush, pea (Pisum sativum) Alaska, and sweet corn (Zea mays) Incredible, were obtained from Ace Hardware (Giddings, TX). Seeds were surface sterilized in the same way as arabidopsis (see above) except in 50 mL of bleach solution. Seeds were grown on PNS either in sterile trays covered with foil at room temperature (pea curvature assay) or in sterile Magenta jars in Percival incubators at 22 °C under yellow light filters (bean and corn auxin growth assays). Different stratification and soaking protocols were tested to determine optimal
parameters for bean and corn growth (Figure 2.1); it is preferable not to stratify the seeds at 4 °C.

2.B. Mutant isolation and nomenclature

Arabidopsis mutants are given descriptive names that are most often represented as three-letter abbreviations followed by a dash and allele number (i.e. pex7-1 for a mutant allele defective in *PEX7*). Gene names are italicized, wild-type genes in uppercase and mutant genes in lowercase fonts (i.e. *PEX7* and *pex7*). Protein names are in regular font, again with wild type in uppercase and mutant protein names in lowercase fonts (i.e. PEX7 and pex7-1).

2.B.1. *pex* mutant isolation

2.B.1.A. Arabidopsis peroxisome biogenesis gene identification

Arabidopsis peroxisome biogenesis *PEROXIN (PEX)* genes were identified by sequence homology to characterized *PEX* genes in yeast and mammals. Previous studies had identified many arabidopsis *PEX* genes through sequence homology or genetics (Table 4.1; Charlton and López-Huertas, 2002; Hayashi et al., 2000; Hu et al., 2002; Lin et al., 1999; Mullen et al., 2001; Schumann et al., 2003; Sparkes et al., 2003; Zolman, 2002; Zolman and Bartel, 2004; Zolman et al., 2000). Despite previous identification of many arabidopsis *PEX* genes, I employed BLAST searches (Altschul et al., 1990) to identify additional genes and examined published cDNA sequences (http://signal.salk.edu/cgi-bin/tdnaexpress; Yamada et al., 2003) to correct annotations of previously identified genes (Table 4.1).

2.B.1.B. IBA response mutant screen

The mutants B292, B491, and B884 were isolated in previous IBA response mutant screens by Bethany Zolman (Zolman, 2002) and Melanie Monroe-Augustus
Figure 2.1. Bean and corn germination assays.
Bean (A) and corn (B) seeds were surface sterilized, incubated in sterile water in the indicated conditions, and grown on PNS at 22 °C under white light. Germination (assayed by embryonic root emergence) was determined after three days.
(Monroe-Augustus, 2004) where EMS-mutagenized seed lines were grown on 20 μM IBA. These mutants were selected for reproducible resistance to root elongation inhibition by IBA.

2.B.1.C. Transfer-DNA insertional disruption alleles

Because efficient means of homologous recombination have not been developed for plants, we relied on insertions of large Transfer-DNA (T-DNA) sequences in or near genes of interest to examine the effects of gene disruption. The Salk Institute T-DNA project characterized over 335,000 T-DNA transformants of arabidopsis Colombia ecotype and identified the location of the insertions in each line by PCR of circularized DNA (Alonso et al., 2003). Arabidopsis gene identification numbers were entered into the Salk T-DNA express website (http://signal.salk.edu/cgi-bin/tdnaexpress) and nearby T-DNA insertion lines were identified and ordered from the ABRC. Upon arrival, the provided T3 or T4 seeds were sterilized, plated, transferred to soil, and grown to adulthood. DNA was extracted from leaves (see 2.D.1.A. below), and PCR was performed using LB1-SALK primer and appropriate gene-specific primers. Appearance of a PCR product indicated the presence of at least one copy of the T-DNA; the resultant PCR product was sequenced to verify the location of the T-DNA insertion. Homozygous lines were identified by PCR of progeny that all contain the T-DNA. If homozygous lines could not be identified, heterozygous lines were utilized for phenotypic analysis.

2.B.1.D. TILLING alleles

Another avenue of reverse genetic allele isolation was through Targeting Induced Local Lesions in Genomes (TILLING; McCallum et al., 2000). These alleles were identified by the arabidopsis TILLING Project from EMS-mutagenized Columbia erecta plants (Till et al., 2003). Using Codons Optimized to Discover Deleterious Lesions (CODDLLe; http://www.proweb.org/input/), we identified regions of the genes of interest
most likely to allow EMS-induced (C to T) missense or nonsense mutations. We
designed fluorescently-labeled oligonucleotides to allow amplification of the chosen
regions, and identification of arabidopsis lines harboring mutations within the region was
performed by the arabidopsis TILLING Project (Till et al., 2003). Once identified, we
examined the mutations using the Project Aligned Related Sequences and Evaluate SNPs
(PARESNP) algorithm (Taylor and Greene, 2003). Using this tool, mutations were
characterized as silent, missense, nonsense, or possibly affecting splicing. Seeds were
ordered from the ABRC and plants were grown to adulthood. Primer sets were designed
to identify plants carrying the different mutations. These were employed on leaf DNA to
identify homozygous lines (if possible) for phenotypic characterization.

2.B.2. ecrl mutant isolation

2.B.2.A. IPrA response screen

A screen for resistance to IPrA was performed on 43,750 ethyl methanesulfonate
(EMS) exposed seeds from 29 pools, 48,000 γ-irradiated seeds from 16 pools, and 18,000
fast neutron-bombarded seeds from 6 pools. Seeds were grown on PNS supplemented
with 5 μM IPrA from a 100 mM ethanol-dissolved stock as described above. After ~8
days, seedlings with aberrantly long roots were removed and aseptically transferred to
PNS for recovery, then grown to maturity in soil. Progeny of these plants were
rescreened on IPrA and IAA. Several mutants were outcrossed to Ler accession for
recombination mapping (see below).

2.B.2.B. Transfer-DNA insertion alleles

Seed lines carrying T-DNA insertions in or near ECR1 or one of two AXR1-like
(AXL) genes were identified and obtained from the ABRC. Plants carrying the insertional
disruptions of interest were identified by DNA extraction and PCR with appropriate
gene-specific primers and LB1-SALK.
2.C. Phenotypic analyses

2.C.1. Root and hypocotyl elongation

Seeds were surface sterilized and grown on PNS and PNS supplemented with various compounds dissolved in 100% EtOH unless otherwise noted. When multiple plates were used in a single experiment, sucrose was added to the PN used for all relevant plates; PNS was aliquoted from the common stock immediately prior to supplement addition. 45 mL of PNS was used per 100 x 100 mm square plate, 100 mL per 150 mm diameter round plate. Plants were grown in Percival incubators under yellow light filters to allay photon destruction of indolic compounds (Stasinopoulos and Hangarter, 1990) at 22 °C unless noted. Plants were grown for 8 days unless otherwise noted, then were removed and roots were measured to the nearest millimeter on a ruler. The mean hypocotyl lengths and standard deviations for each plant type were determined using the Microsoft Excel spreadsheet program.

2.C.2. Lateral root proliferation

Plants were grown on PNS for 4 days to allow even germination and ample primary root elongation, then transferred to supplemented media for an additional 4 days of growth unless indicated. Plants were grown in vertically-oriented media after transfer. All plates were grown under yellow light filters (see above) at 22 °C unless noted. Then, plants were removed and lateral roots, identified as discernable bulges extending out from the primary root, were counted under a dissecting microscope. Tertiary (lateral roots budding from lateral roots) were included in the tallies if present. Data were analyzed as above.

2.C.3. Adventitious root proliferation

Seeds were sterilized, plated on unsupplemented PNS, and grown in white light at 22 °C for one day to induce germination. Plates were then covered with foil and grown
vertically for 4 days. After this time, seedlings were removed and transferred to supplemented and unsupplemented PNS plates and grown vertically at 22 °C for 6 additional days under yellow filtered light. Adventitious roots (root protruding from hypocotyls, the stems between the root and the shoot apical meristem) were then counted under a dissecting microscope. Data were analyzed as above.

2.C.4. Sucrose dependence in darkness

To test for sucrose dependence, seeds were surface sterilized and stratified overnight in 0.1 % agar at 4 °C. Seeds were plated on PN or PNS and placed under white light in a Percival incubator at 22 °C for one day to promote germination. Then, plates were wrapped in two layers of aluminum foil and returned to the Percival incubator at 22 °C for an additional 5 days of growth in darkness unless otherwise noted. Dark-grown (etiolated) plants were removed and the hypocotyls were measured to the nearest millimeter on a ruler. Statistical analysis was performed as above.

2.C.5. Embryonic development

Attempts to visualize embryos in seeds on a Zeiss Axioplan 2 microscope (Thornwood, NY) were unsuccessful, so pex7-1 pex5-1 embryonic development was instead inferred from young seedling phenotypes. More than 112 seeds each of wild type, pex7-1, pex5-1, and three homozygous lines of pex7-1 pex5-1 were grown on PNS under white light for 7 d. These plants were then scored for wild-type, asymmetric, partially fused, or wholly fused cotyledons (embryonic seed leaves).

2.C.6. Seed morphology and viability

Wild type, pex7-1, pex5-1, and pex7-1 pex5-1 were grown on PNS for approximately 14 d., then transferred to soil and grown to maturity. Wild-type and pex7-1 pex5-1 siliques (seed pods) at different stages of maturity were removed, one valve was removed with forceps, and the seeds were photographed on a Leica MZFLII dissecting
microscope (Deerfield, IL). When plants were homogeneously brown and dry, seeds from siliques 4-7 (counting basipetally from the apical terminus of a shoot) were removed, counted, and examined on a dissecting microscope for classification as having a normal plump appearance ("filled") or shriveled abnormal appearance ("abnormal").

2.D. Genetic analysis

2.D.1. Mapping

Mutants were mapped by outcrossing to a different ecotype than the parental strain. F1 seeds resulting from the cross were grown for one generation to allow recombination of the diploid chromosomes. The second generation (F2) was assayed for the mutant phenotype, and polymorphic molecular markers were employed to identify a region linked to the phenotype.

2.D.1.A. Plant DNA isolation

Arabidopsis DNA was isolated by excising a mature leaf, then flash freezing it on dry ice. The tissue was then ground into a fine powder with a chilled pestle. 10 μL of 0.5 M NaOH was added and the tissue was allowed to thaw to room temperature. Next, the tissue in NaOH solution was heated to 100 °C for 30 s to chemically destroy RNA molecules. The mixture was neutralized with 100 μL of 10 mM Tris 1 mM EDTA pH 8 and stored at -20 °C.

2.D.2. pex7-1 kanamycin resistance linkage analysis

Col-0 and pex7-1, and F2 seeds from the first backcross of pex7-1 to Col-0 were surface sterilized and plated on PNS supplemented to 12 μg/mL kanamycin sulfate and were grown under continuous white light at 22 °C for 14 days. All (6/6) Col-0 plants were bleached, indicating kanamycin sensitivity. All (6/6) pex7-1 plants were green, indicating kanamycin resistance. Among progeny of a heterozygous pex7-1 parent, 17/60
(28.3%) were bleached. DNA was extracted from remaining kanamycin-resistant plants from this population and PCR was performed to determine that all but one (42/43) amplified the T-DNA insert. Thus, kanamycin resistance is linked to the T-DNA locus in pex7-1.

2.D.3. Plasmid preparation and transformation

2.D.3.A. Escherichia coli transformation and growth conditions

Chemically competent E. coli strains DH5α, MACH10 (Invitrogen, Carlsbad, CA), and TOP10 (Invitrogen, Carlsbad, CA) were utilized in this study. Cells were stored at -80 °C and transformed under the following conditions.

DH5α cells were thawed on ice and incubated with 2-10 μL DNA for 20 m. Cells were heat shocked by incubation in a 37 °C water bath for 5 m. Cells were then subjected to serial dilution and transferred to selective plates.

MACH10 and TOP10 cells were thawed on ice and incubated with 3-5 μL DNA for 30 m. on ice, then placed in a 42 °C water bath for 30 s., then returned to ice for 2 m. Cells were then suspended in 250 μL of room-temperature SOC medium and recovered on a shaker at 37 °C for 1 h. Transformant mixture and 10-fold and 100-fold dilutions in SOC were plated on 3 prewarmed agar-solidified Lennox Broth (LB) plates with selective antibiotics appropriate to the vector used and returned for overnight growth at 37 °C.

2.D.3.B. Agrobacterium tumefaciens transformation and growth

Agrobacterium strain GV3101 (Koncz et al., 1992) was utilized as a host for plant transformation vectors. Bacteria were stored at -80 °C and grown at room temperature in gentamycin-supplemented LB. Plant transformation vectors were introduced into GV3101 cells by electroporation (Ausubel et al., 1995).
2.D.3.C. *Arabidopsis thaliana* transformation

Plant transformation vectors pBIN19 or 35SpBARN were introduced into plants via Agrobacterium infection. Transformation was performed on mature plants grown in soil at a 5-per-pot density. Prior to transformation, bolts were cut at least once to promote proliferation of inflorescence meristems. Plants were transformed using the floral dip method (Clough and Bent, 1998). After transformation, plants were covered in plastic wrap and placed in darkness overnight, then uncovered and grown in continuous light until maturity.


Progeny (T1) seeds of primary transformants were grown on PN supplemented with an appropriate selective antibiotic. DNA was isolated from leaves of antibiotic-resistant T1 plants and PCR using appropriate primers was performed to identify plants carrying intact and appropriate transgenic DNA. Following PCR confirmation, homozygous lines were then identified in the T3 generation by antibiotic resistance or progeny PCR.

2.D.4. Mutant rescue and overexpression

2.D.4.A  *pex7-1*

The *PEX7* cDNA APZ50H10R was obtained from the Kazusa Stock Center (Asamizu *et al.*, 2000), sequence verified (Lone Star Labs, Houston, Texas), and a 1.3-kb *SmaI/PvuII* fragment was gel-purified and cloned into *SmaI*-digested 35SpBARN vector (LeClere and Bartel, 2001) to generate 35S-*PEX7*. The subcloning boundaries were sequenced with vector-derived oligonucleotides to verify the insert orientation, and 35S-*PEX7* was electroporated into *Agrobacterium tumefaciens* GV3101 (Koncz and Schell, 1986) and transformed into Col-0 and *pex7-1* plants using the floral dip method (Clough and Bent, 1998). T1 seeds were grown on PNS supplemented with 7.5 µg/mL
glufosinate-ammonium (BASTA; Crescent Chemical, Augsburg, Germany); resistant plants were rescued to unsupplemented PN after 8 days and later transferred to soil. The presence of the construct in T1 plants was confirmed by PCR-amplification of genomic DNA with a vector-derived oligonucleotide and an appropriate gene-specific primer. T1 plants confirmed to carry an intact construct were grown to maturity, and T2 progeny were examined for IBA resistance.

2.D.4.B. ecr1

ECRI cDNA clone U13340 (Schmid et al., 2003) was obtained from the ABRC. U13340 DNA was isolated and used as a template for amplification with the modified oligonucleotide primers XhoIECR1 and pUNI51NotI (Table 2.1). Amplification was achieved with 0.3 μL Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA) per 60 μL reaction, 12 reactions total, using the thermocycler CAPS program. Reactions were combined, and DNA was ethanol precipitated for concentration, then resuspended in 20 μL of water. After concentration and size of the PCR product was verified by gel electrophoresis, 1 μL (125 ng) was used in a 10 μL RT ligation reaction with pCR4-TOPO blunt vector (Invitrogen, Carlsbad, CA). Ligation product was transformed into TOP10 E. coli (Invitrogen, Carlsbad, CA), then minipreps of individual white colonies from a carbenicillin X-gal LB plate were digested with EcoRI and NotI to verify the presence of insert. DNA from one colony (ECR1TOPO1) was purified by Qiagen column (Valencia, CA) and sequenced (SeqWright, Houston, TX) to verify the presence of wild-type ECR1 insert.

ECR1TOPO1 DNA was digested with XhoI and NotI, run on a 1% [w/v] agarose gel containing 1 mM guanosine (Gründemann and Schömig, 1996), and the ~1200 base pair band was excised. DNA was extracted from the agarose by centrifugation through glass wool and subsequent ethanol precipitation. 75 ng of this fragment were mixed in an overnight FastLink ligase (Epicentre, Madison, WI) reaction with 320 ng of 35SpBARN
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Table 2.2. Plasmids used in this study continued.

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vector (LeClere and Bartel, 2001) cut with XhoI and NotI. Product was transformed into TOP10 E. coli (Invitrogen, Carlsbad, CA), then plated on LB with kanamycin. Miniprep DNA isolation was performed on the resulting colonies, and one colony was determined by restriction digestion to contain 35S-ECRI. DNA from this colony was Qiagen purified and sequenced (SeqWright, Houston, TX) to verify proper orientation and insert boundaries. 35S-ECRI was transformed into plants as described above.

2.D.5. Northern blot analysis

Tissue was isolated from seedlings grown on 150 mm diameter plates of PNS covered with sterile filter paper to allow tissue collection with minimal medium contamination. Tissue was ground in a mortar and pestle chilled with liquid nitrogen. RNA was extracted either by lithium chloride precipitation (Nagy et al., 1988) or RNeasy column (Ambion, Austin, TX). Concentration was determined by A260 reading on a spectrophotometer. Quality was assessed by the presence of two bright ribosomal RNA bands on a formaldehyde-containing agarose gel. 1 to 5 µg of RNA was loaded onto a 1% agarose formaldehyde gel (Ausubel et al., 1995) and run at ~80 mV for ~45 minutes. The gel was imaged by ethidium bromide fluorescence and transferred to a Hybond nitrocellulose membrane (Amersham, Piscataway, NJ) overnight by capillary action in 20X SSC buffer (Ausubel et al., 1995).

An IAA1 probe was prepared using a cDNA clone (Rogg, 2001) labeled with 32P in a Klenow fragment polymerase (NEB, Beverly, MA)-mediated reaction primed with random 12-mer oligonucleotides (Ausubel et al., 1995). Membrane was pre-hybridized in UltraHyb buffer (Ambion, Austin, TX) in a rotating hybridization oven at 65 °C for at least 1 hour. Freshly-prepared IAA1 probe was denatured at 100 °C for 5 minutes and added to the hybridization solution and incubated as above overnight. After hybridization, the membrane was sequentially washed in 0.1X SSC, 0.1% SDS solution at 65 °C and at room temperature. The membrane was mounted and exposed to X-ray
film in darkness. Additional images were captured on a phosphorous-embedded screen and developed electronically on a Fuji phosphoimager.

2.D.6. Double mutant generation and identification

2.D.6.A. pex7-1 double mutants

Once-backcrossed pex7-1 was cross-pollinated to pex5-1, pex4-1, and pex13-1 mutants also in the Col-0 accession. F2 progeny were grown on PNS at 22 °C in white light and transferred to soil. DNA was extracted from leaves and utilized for PCR-based genotypic analysis.

2.D.6.B. ecr1 double mutants

Once-backcrossed ecr1-1 was cross-pollinated to axr1-12, axr1-3, iaa28-1, cop9-1, ein2, HSAXR3, HSaxr3-1, and ibr5-1 mutants. F2 progeny were grown on PNS at 22 °C in white light and transferred to soil. DNA was extracted from leaves and utilized for PCR-based genotypic analysis. PCR-based genotyping reactions for some alleles are in development.

2.D.7. Microarray analysis

2.D.7.A. Tissue preparation and RNA isolation

Wild-type Col-0 and pxa1-1 mutant (F3 from the third backcross to Col-0) seeds were surface-sterilized and distributed at a density of 750 seeds per plate on sterile filter paper covering PNS on 150 mm diameter round plates (15 plates of each genotype). Additional seeds from the stocks above were plated on PN to verify pxa1 homozygosity. Seedlings were grown at 22 °C under white light. Col-0 and pxa1 grew into seedlings on PNS (though pxa1 seedlings were less robust than wild type); Col-0, but not pxa1, grew on PN, verifying that the pxa1 seed stock was homozygous for the pxa1-1 mutation.
After 8 days, Col-0 tissue was mock-treated by moving seedling-covered filter papers to plates containing 150 mL 1/6 PN with 200 μL ethanol for 2 h. on a benchtop. Subsequently, filter papers were removed, blotted briefly on paper towels, and placed in 50 mL sterile tubes in liquid nitrogen, then stored at -80 °C. pxa1 plants were grown until 10 d. to approximately match wild-type growth at 8 d. These plants were then mock treated as above or treated with a 200 μL volume of ethanol-dissolved IAA or IBA to 10 μM and 100 μM, respectively. Tissue was collected and stored as above. Tissue was homogenized as above and RNA was isolated by lithium chloride precipitation (Nagy et al., 1988).

30 μg of RNA from each of mock-treated Col-0 and pxa1, IAA-treated pxa1, and IBA-treated pxa1 were ethanol precipitated and shipped on dry ice to Texas A&M for tagged cDNA generation and arabidopsis ATH1 ‘whole-genome’ chip (Affymetrix, Santa Clara, CA) hybridization and imaging (Kim Lu and Thomas McKnight, College Station, TX).

2.D.7.B. Data analysis

Data were initially examined using Affymetrix Microarray Suite software. Data were exported to Microsoft Excel spreadsheet files and consolidated. Rows were sorted by Affymetrix gene designation, which were matched to arabidopsis gene identifiers using Excel converter file (http://www-biology.ucsd.edu/labs/schroeder/downloads.html) also sorted by Affymetrix gene designation. Next, data were sorted by Affymetrix Microarray Suite designation of “present,” “meta-present,” or “absent.” Genes with expression designation of “present” in all trials were included in further analysis. Fold change comparisons between data sets were performed by dividing each of the three experimental values by each of the three control values. Mean and standard deviation of the fold changes were calculated from the nine comparison values. Genes in which the standard deviation of the mean fold change were 50% or more of the mean value were
excluded from further analysis. Scatter plots were generated of the remaining data by calculating the mean expression level for experimental and control values.

2.D.8. Mutagenesis

2.D.8.A. Aux/IAA oligomutagenesis

Myc epitope-tagged IAA28 and iaa28-1 genomic clones including the endogenous promoter (KSTH3R1-myc and KSTH3R1iar2-1-myc; Rogg, 2001) were utilized for oligo-directed mutagenesis to introduce a lysine to arginine mutation at amino acid 160. The mutagenic oligo IAA28K160R (Table 2.1) was phosphorylated using T4 DNA kinase (NEB, Beverly, MA). RZ1032 carrying the above plasmids were grown in the presence of helper phage, and resulting single-stranded DNA was purified, annealed to the phosphorylated oligo, and extended using T4 DNA polymerase and ligase cocktail. Resultant DNA was ethanol precipitated and utilized to transform TOP10 DH5α cells. PCR amplification of transformants was used to isolate colonies carrying the K160R mutation. These colonies were grown in 50 mL cultures and DNA was purified by Qiagen column and sequenced with T1N24-19 and T1N24-24 oligos (SeqWright, Houston, TX).

2.D.8.B. PEX7 primer-directed mutagenesis

Col-0 DNA was amplified with PEX7-F and PEX7-R as well as PEX7-F and PEX7-SKL-R oligos (Table 2.1) using Triple Master taq polymerase (Eppendorf, Hamburg, Germany) with an 1.5 min. extension at 72 °C. Products were purified on a 1% [w/v] agarose gel supplemented with guanosine. Amplification products of the expected sizes were extracted from gel fragments using centrifugation through glass wool and ethanol precipitation. Purified DNA products were subcloned into pCR4-TOPO blunt vector and transformed into TOP10 E. coli (Invitrogen, Carlsbad, CA) cells. DNA was isolated from minipreps of carbenicillin-resistant colonies and digested with EcoRI

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and KpnI to identify colonies carrying the desired insert. Sequencing with T3 and T7 vector-derived oligonucleotides (Table 2.1) revealed a PEX7 colony with the proper insert sequence and orientation; however, a PEX7-SKL colony was in the reverse orientation. Therefore, colony PCR of additional PEX7-SKL colonies was performed using M13-F and PEX7-1 oligos (Table 2.1) to identify colonies with the PEX7-SKL insert in the desired orientation.

PEX7 and PEX7-SKL in pCR4-TOPO were digested with DraI and NotI restriction endonucleases and subcloned into SmaI NotI digested 35SpBARN (LeClere and Bartel, 2001) using FastLink DNA Ligase (Epicentre, Madison, WI) overnight at room temperature. Ligated plasmid was transformed into TOP10 E. coli (Invitrogen, Carlsbad, CA) and plated on LB with kanamycin. DNA was extracted from colonies by miniprep (see above) and tested for insert. Colonies apparently carrying PEX7 or PEX7-SKL were utilized for Qiagen column DNA purification and sequencing with vector-derived oligonucleotides (SeqWright, Houston, TX).

2.E. Visualization of matrix protein import by green fluorescent protein

2.E.1. PTS2-GFP construct generation

A PTS2-tagged GFP was created by amplifying the 5´ 147 bp of arabidopsis PED1 thiolase (Hayashi et al., 1998) clone U09045 (Yamada et al., 2003) that encode the N-terminal region containing the PTS2 signal sequence and the signal sequence cleavage site (Johnson and Olsen, 2003). Oligonucleotides PTS2XbaI and PTS2Bam2 were used for PCR amplification of U09045 template DNA with Pfu Turbo DNA polymerase (Stratagene). The resultant 175-bp blunt-ended product was gel-purified and subcloned into EcoRV-cut pBluescript KS+ (StrataGene, La Jolla, CA) and sequenced to verify the absence of mutations. From this construct, the 155-bp XbaI/BamHI fragment was gel-purified and cloned into XbaI/BamHI-digested CD3-326 (Davis and Vierstra, 1998)
downstream of the 35S promoter and in frame with the gene encoding enhanced GFP. From this construct, a 2 kb EcoRI/HindIII product that included the 35S promoter and PTS2-GFP fusion was gel-purified and cloned into EcoRI/HindIII-digested pBIN19 plant transformation vector (Bevan, 1984) to yield PTS2-GFP. Cytoplasmic eGFP (unmodified CD3-326) and PTS2-GFP were transformed into Agrobacterium and then Col-0, pex7-1, and pex5-1 plants as described above.

2.E.2. Transgenic isolation

Col-0, pex5-1, and pex7-1 T1 seeds were screened on PNS with 13 μg/mL kanamycin sulfate from a water-dissolved stock, and kanamycin-resistant Col-0 and pex5-1 T1 plants were rescued to PN plates after 9 days. The existence of a kanamycin resistance gene in the T-DNA insert in pex7-1 T1 plants necessitated isolating transformants by screening for GFP fluorescence on a Leica MZFLIII dissecting scope, and GFP-expressing pex7-1 plants were rescued as above. To monitor GFP localization, T2 seedlings were mounted on slides, and GFP fluorescence in root tips of 3-day-old seedlings grown in white light suspended in 0.1% agar and root hairs of 7-day-old seedlings grown in white light on PNS were examined by using a Zeiss Axioplan 2 fluorescence microscope equipped with a narrow-band GFP filter set (41020, Chroma Technology, Rockingham, VT).

Col-0 plants expressing PTS1-GFP, a CD3-326 construct mutagenized to introduce the PTS1 signal sequence SKL to the extreme C-terminus of eGFP (the construct GFP-SKL from Zolman and Bartel, 2004), were crossed to pex7-1 and pex5-1 plants. and IBA-resistant F2 progeny were genotyped using PCR amplification for the pex7-1 and pex5-1 mutations. Lines segregating GFP fluorescence were identified and used for analysis as described above.
2.F. Western blot analysis

2.F.1. Anti-PEX7 antibody development

Regions of PEX7 appropriate for peptide antibody development were identified by Bethyl Laboratories (Montgomery, TX) and utilized to develop two potential rabbit α-arabidopsis PEX7 antibodies. Peptides were synthesized, then conjugated to keyhole limpet hemocyanin (KLH) and injected into rabbits; antibodies were affinity purified from rabbit serum (Bethyl Laboratories, Montgomery, TX).

2.F.2. Gels, transfer, blotting, and visualization

2.F.2.A. α-PEX5 and α-thiolase antibodies

Protein was extracted from entire 2-day-old Col-0, pex7-1, and pex5-1 seedlings grown aseptically in water under white light and 3-day-old Col-0, pex7-1, pex5-1, and pex7-1 pex5-1 seedlings grown in 0.5% sucrose under white light. Germination is slightly delayed by sucrose, so the different chronological ages represent equivalent developmental stages. 25 seedlings with emerged embryonic roots (radicles) were selected from each genotype, frozen, ground with a pestle, and suspended in one volume of extraction buffer (0.1 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS). Samples were briefly centrifuged to pellet debris and supernatants were heated for 10 minutes at 80 °C. Protein extracts were subjected to SDS-PAGE on NuPAGE 10% Bis-Tris gels (Invitrogen, Carlsbad, CA) with Cruz size standards (Santa Cruz Biotechnology, Santa Cruz, CA).

Protein was transferred to Hybond enhanced chemiluminescence nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 hour at 24 V. Membranes were blocked in 5% powdered milk in TTBS buffer (Ausubel et al., 1995) for at least 2 hours, then incubated with rocking at 4 °C for at least 13 hours in a 1:1000 dilution of rabbit anti-plant thiolase antibody (Kato et al., 1996) or a 1:200 dilution of a
rabbit anti-arabidopsis PEX5 antibody (Zolman and Bartel, 2004) in 5% milk TTBS. Membranes were washed four times in 5% milk TTBS, then incubated for at least 1 hour at room temperature in a 1:500 dilution of a horseradish peroxidase-conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology). Membranes were washed as before and visualized using LumiGLO reagent (Cell Signaling, Beverly, MA).

2.F.2.B. α-PEX7 antibody

50 seeds each of Col-0, pex7-1 F4c-1, Col-0 35S-PEX7 #1 T3, and Col-0 35S-PEX7 #3 T3 were surface sterilized and grown in water in 1.5 mL tubes under continuous white light for 2 days. After this time, the water volume in each tube was adjusted to 50 μL and samples were stored at -80 °C.

Samples were thawed on ice water, 50 μL of LLB buffer was added to each, and the samples were homogenized with a motorized pestle until froth filled each tube, then samples were returned to ice water. Samples were spun at 13,200 rpm in a tabletop centrifuge for 5 min., then the supernatants were removed to new tubes. 10 μL 0.5 M DTT dissolved in water was added to each sample, then the samples were heated to 100 °C for 5 min. 15 μL of each sample was loaded into each of four replicate lanes split between two 15-well 10% Bis-Tris NuPAGE protein electrophoresis gels (Invitrogen, Carlsbad, CA). 5 μL of Cruz size standard marker (Santa Cruz Biotechnology, Santa Cruz, CA) and 15 μL of prestained protein marker (NEB, Beverly, MA) were loaded adjacent to each of the 4 replicates. Electrophoresis was performed at 110 mA (~160 mV) for 1 h. in MOPS buffer containing antioxidant in the inner chamber.

Transfer, blocking, and antibody hybridization were performed as above with the following exceptions. Each of the two primary antibodies was incubated with a membrane at 1:500 and 1:5,000 dilutions. Secondary antibody was visualized with LumiGlo Enhanced reagent (Cell Signaling, Beverly, MA).
2.F.2.C. α-CUL1 antibody

25 seeds each of Col-0, axr1-12, axr1-3, ecr1-1 (backcrossed to Col-0), and dark-colored cop9 seeds were surface sterilized and grown in water in 1.5 mL tubes under continuous white light for 3 days. After this time, the water volume in each tube was adjusted to 25 μL and samples were stored at -80 °C.

Samples were thawed on ice water, 25 μL of LLB buffer (Ausubel et al., 1995) was added to each, and the samples were homogenized with a motorized pestle until froth filled each tube, then samples were returned to ice water. Samples were spun at 13,200 rpm in a tabletop centrifuge for 5 min., then the supernatants were removed to new tubes. 5 μL 0.5 M DTT dissolved in water was added to each sample, then the samples were heated to 100 °C for 5 min. 25 μL of each sample was loaded a 12-well 10% Bis-Tris NuPAGE protein electrophoresis gel (Invitrogen, Carlsbad, CA). 5 μL of Cruz size standard marker (Santa Cruz Biotechnology, Santa Cruz, CA) and 15 μL of prestained protein marker (NEB, Beverly, MA) were loaded adjacent to each of the 4 replicates. Electrophoresis was performed at 110 mA (~160 mV) for 1.5 h. in MOPS buffer containing antioxidant in the inner chamber. Transfer, blocking, and antibody hybridization were performed as with α-PEX7 above except that a 1:5,000 dilution of primary antibody was used.

2.G. Quantitative reverse-transcription real-time PCR

2.G.1. RNA isolation and reverse-transcriptase PCR

Seven-day-old Col-0, pex7-1, pex5-1, and pex7-1 pex5-1 seedlings grown under white light on PNS were frozen in liquid nitrogen and ground in a chilled mortar and pestle. RNA was extracted using RNeasy Plant Mini Kits (Qiagen, Valencia, CA). Following OD_{260} quantification and electrophoresis to ensure the absence of RNA degradation, each sample was treated with DNaseI (Amplification Grade, Roche Applied
Science, Indianapolis, IN) and 0.3 μg was reverse-transcribed in a 20 μL volume using a random hexamer primers and SuperScript III polymerase (Invitrogen, Carlsbad, CA). Controls lacking reverse transcriptase were performed for each sample.

2.G.2. Real-time PCR

Quantitative real-time reverse-transcription PCR using an ABI Prism 7000 Sequence Detection System was performed on triplicate 25 μL reactions containing 2 μL cDNA, 0.5 μM each primer, and 0.2 μM probe in Taqman universal PCR Master Mix (Applied Biosystems, Foster City, CA). Primers for PEX7 were PEX7-QRTF and PEX7-QRTR and a 5´ 6-FAM-labeled, 3´ MGBNFQ (minor groove-binder/nonfluorescent quencher) probe. Primers for the APRT control were APRT-F and APRT-R and a 5´ 6-FAM-labeled, 3´ MGBNFQ-quenched probe. APRT amplification was used to normalize PEX7 levels using a comparative C_t method (ABI Prism 7700 Sequence Detection System User Bulletin #2, http://www.appliedbiosystems.com). Control reactions without reverse transcriptase amplified later than experimental samples.
Chapter 3: Peroxisomal protein import is necessary for plant development and IBA response

3.A. Interaction among peroxisomal targeting signal receptors

Peroxisomes are organelles housing diverse and vital processes. Plant peroxisomes contain enzymes for photorespiration (Liepman and Olsen, 2001; Reumann, 2002) and are the primary, if not exclusive, site of fatty acid β-oxidation (Gerhardt, 1992; Graham and Eastmond, 2002; Kindl, 1993). In addition, plant peroxisomes are necessary for jasmonic acid (JA) biosynthesis (Wasternack and Hause, 2002) and are implicated in the conversion of indole-3-butyric acid (IBA) into the active auxin indole-3-acetic acid (IAA; Bartel et al., 2001; Woodward and Bartel, 2005b; Zolman et al., 2000).

Peroxisome matrix proteins must be imported from the cytosol because peroxisomal proteins are encoded by nuclear genes. Import of matrix proteins is accomplished by an array of PEROXIN (PEX) proteins that function in matrix protein import or general peroxisome assembly (Chapter 1). Over 30 pex mutants have been identified in yeast and mammals; in plants, only pex2 (Hu et al., 2002), pex5 (Zolman et al., 2000), pex6 (Zolman and Bartel, 2004), pex10 (Schumann et al., 2003; Sparkes et al., 2003), pex14 (Hayashi et al., 2000), and pex16 (Lin et al., 1999) arabidopsis mutants have been isolated. However, plausible arabidopsis homologs for many of the PEX genes remaining to be characterized have been identified through sequence homology (Charlton and López-Huertas, 2002; Mullen et al., 2001).

Two sequences sufficient to signal matrix protein import into peroxisomes have been identified. One is the extreme C-terminal amino acid sequence Ser-Lys-Leu (SKL), or a conserved variant, designated Peroxisomal Targeting Signal Type 1 (PTS1; Gould et al., 1989; Mullen, 2002; Neuberger et al., 2003; Reumann, 2004). The PEX5 receptor

* Portions of this chapter have been previously published (Woodward and Bartel, 2005a).
recognizes and binds cytosolic PTS1-containing proteins. Mutations in human \textit{PEX5} can cause the peroxisome biogenesis disorders Zellweger syndrome and neonatal adrenoleukodystrophy (Dodt et al., 1995). The PEX5-PTS1 complex binds a PEX14-PEX13 receptor complex at the peroxisome membrane (Albertini et al., 1997) and is translocated into the peroxisome matrix (Dammai and Subramani, 2001) in a process dependent on PEX2, PEX10, and PEX12 (Chang et al., 1999; Dodt and Gould, 1996). In the peroxisome matrix, PEX5 releases its cargo and is recycled to the cytosol (Dammai and Subramani, 2001) in a process dependent on PEX1, PEX4, PEX6, and PEX22 (Collins et al., 2000).

An alternate sequence is the N-terminal Peroxisomal Targeting Signal Type 2 (PTS2) composed of Arg-Leu-(X)$_5$-His-Leu, or a number of variants, within the first \(\sim 30\) amino acids (Flynn et al., 1998; Osumi et al., 1991; Reumann, 2004). PEX7 is the receptor for PTS2-containing proteins (Rehling et al., 1996). Mutations in human \textit{PEX7} can cause the peroxisome biogenesis disorders Refsum disease (van den Brink et al., 2003) and rhizomelic chondrodysplasia punctata (Braverman et al., 1997; Motley et al., 1997; Purdue et al., 1997). In \textit{Saccharomyces cerevisiae}, Pex7p interacts with Pex18p and Pex21p, two functionally-redundant proteins necessary for PTS2 protein import (Purdue et al., 1998). In \textit{Neurospora crassa} and \textit{Yarrowia lipolytica}, Pex18p and Pex21p are replaced with the single Pex7p docking protein Pex20p (Einwächter et al., 2001; Sichting et al., 2003).

Mammals and plants lack apparent \textit{PEX18}, \textit{PEX20}, and \textit{PEX21} orthologs. Mammalian PEX7 function instead depends on interaction with an isoform of PEX5 (PEX5L; Braverman et al., 1998) containing a PEX7-binding domain (Dodt et al., 2001; Matsumura et al., 2000). Whereas most PEX5 mutants are defective in PTS1 protein import, a PEX5 mutant Chinese hamster ovary cell line is specifically deficient in PTS2 protein import because of an inability to bind PEX7 (Matsumura et al., 2000). Similarly, arabidopsis PEX7 interacts with PEX5 \textit{in vitro} (Nito et al., 2002), and \textit{in vitro} import of a
PTS2 protein into *Cucurbita pepo* glyoxysomes is enhanced by PTS1 protein addition (Johnson and Olsen, 2003). Indeed, a role for PEX7-PEX5 interaction in arabidopsis PTS2-protein import has been proposed (Sparkes and Baker, 2002). The dependence of PEX7 on PEX5 interaction in some organisms suggests a convergence of function carried to the extreme in *Caenorhabditis elegans*, which lacks PEX7 entirely and employs a PTS1 on proteins targeted by PTS2 in other organisms (Gurvitz et al., 2000; Motley et al., 2000).

Insertional null mutations in arabidopsis *pex2* (Hu et al., 2002) and *pex10* (Schumann et al., 2003; Sparkes et al., 2003) confer embryo lethality, revealing the necessity of peroxisome function for early plant development. Plants heterozygous for a *pex10* knockout allele bear ~20% abnormal seeds that fail to germinate (Sparkes et al., 2003). The aborted seeds remain white rather than proceeding to green and contain embryos arrested at the late globular or heart stages of development (Schumann et al., 2003). As peroxisomes are necessary for JA biosynthesis (Wasternack and Hause, 2002) and implicated in the conversion of IBA into IAA (Bartel et al., 2001; Woodward and Bartel, 2005b), deficiencies in JA and IAA have been suggested as possible causes for *pex10* mutant lethality (Sparkes et al., 2003). In addition, a double mutant defective in two peroxisomal acyl-CoA oxidase genes is embryo lethal (Rylott et al., 2003), suggesting that β-oxidation is an essential function for which peroxisomes are required during embryogenesis.

3.A.1. **PEX5 and PEX7 interact in mammals**

3.A.1.A **PEX5 isoforms**

The PTS1 receptor PEX5 is present in two forms in mammals, a short form called PEX5S and a long form called PEX5L (Braverman et al., 1998). The size difference results from an alternate splicing event that excludes one *PEX5L* exon from the *PEX5S*
transcript (Braverman et al., 1998). The functional difference between PEX5 isoforms appears to be limited to PEX7 interaction: PEX5L is competent for PEX7 binding, but PEX5S is not (Braverman et al., 1998). Indeed, the region present specifically in PEX5L is conserved among proteins that interact with PEX7 from other organisms (Einwächter et al., 2001). A Chinese hamster ovary cell line that is specifically deficient in PTS2 protein import into peroxisomes has a serine to phenylalanine missense mutation in this region of PEX5L ((Matsumura et al., 2000).

To date, nothing is published describing the relative timing of PEX5L and PEX5S expression, though PEX5S was the first to be isolated as a cDNA. However, these PEX5 isoforms could play different roles in mammalian development. Differential expression of PEX5 isoforms would allow regulation of PTS2 protein import, enabling control of the PTS1:PTS2 protein ratio within peroxisomes.

3.A.2. PEX7 and PEX5 may interact in other vertebrates

3.A.2.A. Zebrafish PEX5 isoforms may exist

Like mammals, the vertebrate fish Danio rerio PEX5 gene appears to have the capability to encode alternate isoforms. Annotated zebrafish cDNAs for PEX5 correspond to the PEX5S isoform of mammalian PEX5 (Figure 3.1). However, I detected a region between zebrafish PEX5 exons 7 and 8 that, if included in the PEX5 transcript, could encode a PEX5L protein that would include the conserved PEX7-binding domain (Figures 3.2 and 3.3). Nucleotides adjacent to the potential in-frame exon are appropriate splice site donor and acceptor sequences. If a cDNA for zebrafish PEX5L could be isolated, zebrafish could prove a useful model for studying the specific roles of PEX5 isoforms in vertebrate development.

In addition to PEX5, I identified the PEX7 protein in zebrafish (AAH90898). Further supporting a role for PEX7 in bony fish peroxisome biogenesis, I was able to
Figure 3.1. Organization of PEX5 proteins.

A, Schematic showing domain architecture of PEX5 proteins. The bracket above PEX5 is the region shown in B, the asterisk denotes the Ser to Leu mutation indicated in B.

B, Partial alignment of PEX5 from human (Hs), zebrafish (Dr), Arabidopsis (At), rice (Os), fly (Dm), yeast (Sc), and roundworm (Ce). pex5-1 contains a missense mutation in a conserved serine (Zolman et al., 2000) needed for PEX7 interaction in PEX5 and PTS2-containing protein import into peroxisomes in Chinese hamster (Matsumura et al., 2000). Note the absence of this domain in CePex5 and ScPex5 proteins; C. elegans does not contain PEX7 or a PTS2 import pathway (Gurvetz et al., 2000; Motley et al., 2000) and S. cerevisiae PEX5 does not interact with PEX7. DrPex5L and AtPex5S are hypothetical; a short form of OsPex5 could also exist. Sequences were aligned with the MegaAlign program (DNA Star, Madison, WI) using the ClustalW method; residues identical in three or more sequences are shaded black, residues chemically similar in three or more sequences are shaded gray. PPR and TPR domains after Zolman et al. 2000.
Figure 3.2. Potential PEX7 binding domains.

Regions similar to the human (Hs) PEX5 domain sufficient for PEX7 interaction (Dodt et al., 2001) were identified and aligned by hand using the MegAlign program (DNA Star, Madison, WI). Residues shaded black are identical in a majority of sequences; residues shaded grey are chemically similar in a majority of sequences. Dr is the bony fish Danio rerio; Gg is the bird Gallus gallus; Ci is the tunicate Ciona intestinalis; At is the dicotyledonous plant Arabidopsis thaliana; Os is the monocotyledonous plant Oryza sativa; Ld is the protist Leishmania donovani; Tb is the protist Trypanosoma brucei; Dd is the protist Dictostelium discoideum; Sc is the fungus Saccharomyces cerevisiae; Yl is the fungus Yarrowia lipolitica. ScPEX18, ScPEX21, and YlPEX20 are PEX7-interacting proteins. Ma1360 is the 1360 protein from the archaeabacterium Methanosarcina acetivorans. Note that ScPEX5 does not directly interact with PEX7, and Ma1360 does not appear to be a PEX5 protein.
identify potential PTS2 cargo in zebrafish. The protein alkylidihydroxyacetonephosphate synthase (ADS) is targeted to peroxisomes by a PTS2 signal sequence in humans, but is targeted by a PTS1 in the nematode worm *C. elegans* that lacks the PTS2 pathway and *PEX7* (Motley et al., 2000). I identified a zebrafish ADS protein by sequence identity (Figure 3.3). As in humans, the zebrafish ADS bears an N-terminal PTS2 signal sequence rather than a C-terminal PTS1 (Figure 3.3). Similarly, human and zebrafish 3-ketoacyl-CoA thiolases both contain PTS2 signal sequences (Figure 3.4).

3.A.2.B. **PEX5 and PEX7 may interact in chickens**

Because mammals and bony fishes may share the capability to encode both PEX7-binding and nonbinding isoforms of PEX5, I sought to determine whether birds, another class of vertebrates, preserved this capability. A BLAST search of human PEX5 against the chicken (*Gallus gallus*) predicted proteins revealed a PEX5 isoform (cDNA ID 53126788) with a domain similar to the PEX7-binding region of mammalian PEX5L (Figure 3.2). Because the protein sequence is derived from a cDNA and the genomic sequence was not identified, it remains unclear whether the chicken genome is capable of encoding a non-PEX7-binding isoform of PEX5. However, the presence of the PEX7-binding isoform is consistent with the hypothesis that PEX5-PEX7 interaction is important for vertebrate development. In addition, I identified chicken PEX7 (cDNA ID 41633907).

3.A.2.C. **PEX5 and PEX7 may interact in tunicates**

Mammals, bony fishes, and birds contain the genetic capacity to encode a PEX5 isoform with a region that may interact with the PTS2 receptor PEX7 (Figure 3.2). Because the PEX7-binding PEX5 isoform seems to be conserved among vertebrates, I examined the tunicate *Ciona intestinalis*, a member of the urochordates closely related to vertebrates (Adoutte et al., 2000). I identified a tunicate cDNA capable of encoding a
Figure 3.3. Animal alkylidihydroxyacetonephosphate synthases.
Proteins similar to the human peroxisomal enzyme alkylidihydroxyacetonephosphate synthase (HsADS) were identified in the bony fish *D. rerio* (DrADS), the arthropod *Drosophila melanogaster* (DmADS), and the roundworm *C. elegans* (CeADS), and these proteins were aligned using the MegAlign program (DNA Star, Madison, WI) ClustalW algorithm. Residues shaded black are identical in a majority of sequences; residues shaded grey are chemically similar in a majority of sequences. Boxed residues are potential peroxisomal signal sequences: N-terminal boxes indicate PTS2 sequences recognized by the PEX7 receptor; C-terminal boxes indicate PTS1 sequences recognized by the PEX5 receptor.
Figure 3.4. 3-ketoacyl-CoA thiolases.

Proteins similar to the human peroxisomal enzyme 3-ketoacyl-CoA thiolase (HsKAT) were identified in the bony fish D. rerio (DrKAT), the arthropod D. melanogaster (Dm thiolases), the annelid worm C. elegans (CeKAT), and the dicotyledonous plant A. thaliana (At PED1 thiolase). These proteins were aligned using the MegAlign program (DNA Star, Madison, WI) ClustalW algorithm. Residues shaded black are identical in a majority of sequences; residues shaded grey are chemically similar in a majority of sequences. Boxed residues are potential peroxisomal signal sequences; N-terminal boxes indicate PTS2 sequences recognized by the PEX7 receptor; C-terminal boxes indicate PTS1 sequences recognized by the PEX5 receptor.
PEX5 isoform containing the PEX7 interaction domain (Figure 3.2). The tunicate genome is not fully sequenced, and genomic DNA encoding PEX5 was not identified. Therefore, it appears that PEX5-PEX7 interaction is ancestral to most vertebrate lineages, and at least mammals and fishes have the capability to encode an additional non-PEX7-interacting form of PEX5. Whether expression of a PEX5 isoform that does not bind PEX7 is conserved among birds and tunicates remains unresolved.

3.A.3. PTS2 pathway absent in a roundworm

Mammals and plants share a similar pathway for PTS2 protein entry into peroxisomes that depends on the PTS1 receptor, while fungi utilize parallel pathways for the differently targeted proteins. However, certain organisms do not appear to utilize the PTS2 pathway at all. For example, the nematode worm *Caenorhabditis elegans* lacks the PEX7 receptor (Gurvitz et al., 2000; Motley et al., 2000). Further, PTS2-targeted proteins have not been identified in *C. elegans*, and proteins targeted by PTS2 in other organisms bear PTS1 targeting signals in the nematode (Gurvitz et al., 2000; Motley et al., 2000). Further, if a synthetic PTS2-targeted protein is expressed in *C. elegans*, the protein is mislocalized to the cytoplasm (Motley et al., 2000).

3.A.4. PTS2 pathway is not utilized in flies

As with roundworms, the PTS2 does not appear to be utilized in flies. The *Drosophila melanogaster* genome contains sequence that appears to encode PEX7, however, no PTS2 proteins have been identified (Motley et al., 2000). Like roundworms, drosophila employs a PTS1 signal sequence on peroxisomal proteins targeted by PTS2 in other organisms (Figures 3.4 and 3.5). Further, the drosophila genome encodes a single possible PEX5 transcript (the gene contains no introns) that encodes a protein lacking the PEX7 interaction domain (data not shown). Also, there are no genes with significant sequence homology to PEX18, PEX20, or PEX21 proteins that are involved in parallel
PEX7 peroxisome association in fungi (data not shown). Thus it appears that flies and nematodes have become solely reliant on the PTS1 targeting system.

3.A.5. PEX5 and PEX7 may interact in some protozoans

Protozoans are a diverse assemblage of organisms divergent from other eukaryotes. I searched for PEX5 in *Leishmania donovani, Trypanosoma brucei,* and *Dictyostelium discoideum* and identified isoforms containing the PEX7 interaction domain (Figure 3.2). In addition, I identified PEX7 in *D. discoideum* (EAL67514). Further, 3-ketoacyl-CoA thiolase bears a PTS2 targeting sequence in *D. discoideum* (Motley et al., 2000). Therefore, it is likely that interaction between peroxisomal targeting signal receptors is ancient and has been lost or modified in some organisms. In addition, because only the PEX7-interacting region-containing PEX5 isoform was identified in three species, it appears likely that the ability to express a PEX5 isoform lacking the region is a strategy that was not present in the ancestor of these protists and higher eukaryotes.

3.A.6. PEX5 and PEX7 function independently in fungi

Implied mechanistic similarities between vertebrate and protist PEX7 and PEX5 proteins suggest an ancient origin for interaction between these peroxisomal targeting signal receptors (Figure 3.5). However, fungi, which diverged from a common lineage with animals after the divergence of plants (Adoutte et al., 2000), employ a different strategy for import of PTS1 and PTS2 proteins. *Saccharomyces cerevisiae* contains both PEX5 and PEX7; these proteins function similarly in yeast as in other organisms. However, the mechanism of PTS2 protein import is very different in yeast. Whereas mammalian and plant PEX7 depends on interaction with PEX5 to deliver PTS2 protein cargo to the peroxisome, budding yeast Pex7p enters the peroxisome independently of Pex5p (Purdue et al., 1998).
Figure 3.5. Evolutionary relationships and peroxisome biogenesis strategies.
The top panel summarizes the evolutionary relationships between the organisms discussed in the text. The phylogenetic tree represents relationships of based upon a composite of several proteins created by Baldauf (1999). The letters A, B, and C refer to the alternate peroxisomal protein import strategies illustrated in the lower panel. Strategy A is utilized by organisms that lack PEX7, PTS2 proteins, and a PEX7 interaction domain in PEX5. Strategy B is utilized by organisms that contain PEX7, PTS2 proteins, and a PEX5 isoform with the PEX7 interaction domain. A and B together indicate potential alternate splicing of PEX5 to generate both PEX7-interacting and non-interacting proteins. Strategy C, utilized by fungi, employs parallel machinery for PEX7 and bound PTS2 cargo to dock with the peroxisome using the proteins PEX18, PEX20, or PEX21. Pink letters represent strategies first examined in this work, blue letters represent previously-characterized strategies (see text for references).
*S. cerevisiae* Pex7p docks at the peroxisome via peroxins not shared with other organisms. Docking is dependent on Pex18p and Pex21p, functionally redundant proteins that bear the PEX7 binding sequence similar to those present in mammalian PEX5L and plant PEX5.

Likewise, the fungi *Neurospora crassa* and *Yarrowia lipolytica* contain another peroxin, Pex20p, that bears the PEX7 interaction motif (Einwächter et al., 2001; Sichting et al., 2003). These fungi, like *S. cerevisiae*, contain a Pex5p protein lacking the PEX7 interaction motif. Thus, it appears that in fungi PEX7 is recognized by specialized peroxins for docking with the peroxisome, making PTS2-protein import parallel to and independent of PTS1-protein import.

3.A.7. **PEX5 and PEX7 may interact in plants**

The arabidopsis *PEX5* gene is necessary for peroxisomal function (Zolman et al., 2000). The encoded protein contains sequences with homology to the PEX7-interacting domain from vertebrate PEX5L (Figure 3.1). Further, potential PEX7-interacting domains are present in PEX5 of dicotyledonous plants including potato and tobacco and the monocotyledonous plant rice (Figure 3.2 and data not shown). Thus, this domain is present across diverse plant phyla and may be generally important for plant development. However, unlike the case in mammals, arabidopsis PEX5 appears to be present only in a PEX5L-like form. No cDNAs for a *PEX5* lacking this region have been described. Further, examination of the arabidopsis *PEX5* gene reveals that simply excluding exon 7, which encodes the PEX7-interaction domain (as in mammalian PEX5S), would eliminate two nearby pentapeptide repeat domains involved in other PEX5 functions (only one pentapeptide domain is excluded in mammalian PEX5S; Figure 3.1). RT-PCR experiments could be employed to determine whether exon 7 can be skipped or an alternate exon exists in arabidopsis.
3.B. Peroxisomal targeting signal receptor mutants

3.B.1. *pex7-1* is deficient in peroxisomal processes and PTS2 import

Sequence similarity searches have been employed to identify *Atlg29260* as arabidopsis *PEX7* (Schumann et al., 1999). The single-copy gene encodes a protein ~40 and ~59 percent identical to PEX7 from *S. cerevisiae* and humans, respectively, that is largely comprised of WD40 domains (Figure 3.6). To determine the role of PEX7 in plant development, I isolated the *pex7-1* mutant from the Salk Institute T-DNA insertion collection (Alonso et al., 2003) and identified a single kanamycin resistance-linked T-DNA 95 bp upstream of the start codon, within the *PEX7* 5' untranslated region.

Like previously-described arabidopsis mutants with peroxisomal defects (Zolman and Bartel, 2004; Zolman et al., 2001a; Zolman et al., 2001b; Zolman et al., 2000), *pex7-1* displays reduced sensitivity to exogenously supplied IBA (Figures 3.7, 3.8, and 3.9). In addition, the mutant is resistant to an analogous synthetic proto-auxin, 2,4-dichlorophenoxybutyric acid (2,4-DB; Figure 3.7), which is presumed to be converted in peroxisomes to the active derivative 2,4-dichlorophenoxyacetic acid (2,4-D; Hayashi et al., 1998). However, auxin response is not generally compromised in the mutant because *pex7-1* retains wild-type sensitivity to exogenous IAA (Figures 3.7 and 3.8). The IBA resistance of *pex7-1* is rescued by overexpression of wild-type *PEX7* (Figure 3.9), indicating that the phenotype observed results from reduced *PEX7* function.

Some peroxisomal mutants produce fewer lateral roots than wild type even without exogenous auxin (Zolman and Bartel, 2004; Zolman et al., 2001b). Any lateral root production defect in *pex7-1* on unsupplemented medium is weak (Figures 3.8 and 3.9), though reproducible. In addition, *pex7-1* forms fewer lateral roots than wild type after IBA treatment (Figure 3.8). *pex7-1* responds normally to induction of lateral roots by IAA (Figure 3.8).
Figure 3.6. Organization of PEX7 proteins.

A, Schematic showing domain architecture of PEX7 proteins. B, PEX7 is the single arabidopsis (At) homolog of S. cerevisiae (Sc) and human (Hs) PEX7, and is highly similar to rice (Os) TIGR gene temporary ID 8351.t01315 (OsPEX7). Arrowhead denotes the upstream location of the T-DNA insertion in pex7-1; arrows depict missense conversion in pex7 alleles obtained from TILLING. Sequences were aligned with the MegAlign program (DNA Star, Madison, WI) using the ClustalW method; residues identical in three or more sequences are shaded black, residues chemically similar in three or more sequences are shaded gray. WD-40 domains determined by Pfam (Bateman et al., 2002).
Figure 3.7. IBA and 2,4-DB sensitivity is reduced in *pex7-1*.
Seedling root lengths were measured after eight days of growth on PNS medium or PNS supplemented with the indicated compound at 22 °C under yellow light filters. Homozygous progeny of independent *pex7-1* backcrosses are shown. IAA is indole-3-acetic acid; IBA is indole-3-butyric acid; 2,4-DB is 2,4-dichlorophenoxybutyric acid; MeJA is methyljasmonate. Bars represent means + standard deviations; *n* ≥ 11.
Figure 3.8. IBA and 2,4-DB sensitivity is reduced in pex7-1.
Seedlings were grown at 22 °C under yellow light filters for 5 days on PNS, then transferred to PNS and PNS supplemented with the indicated compounds for an additional 3 days of growth. Root growth after transfer was measured (A) and lateral roots were counted using a dissecting microscope (B). Homozygous progeny of independent pex7-1 backcrosses are shown. Bars represent means + standard deviations; $n \geq 9$. 

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Figure 3.9. *pex7*-1, *pex5*-1, and *pex7*-1 *pex5*-1 are deficient in peroxisomal processes. 
A, Root elongation on IBA. Root lengths of 8-day-old plants grown on sucrose-supplemented medium with or without addition of 5 μM IBA under yellow light filters are shown. B, Percent root elongation on 5 μM IBA versus hormone-free medium. Plants were grown as in panel A. C, Lateral root number. Lateral roots of 9-day-old plants grown on PNS under yellow light filters were counted under a dissecting microscope. D, Hypocotyl elongation in darkness without sucrose. Plants were grown one day in white light, then grown in darkness for 5 additional days. Bars represent means ± standard deviations, n ≥ 6 in A, n ≥ 10 in B, C, and D. Asterisks indicate values significantly different from wild type (P < 0.01; one-tailed t-test assuming unequal variance). E, *pex7*-1 *pex5*-1 does not develop after germination without sucrose in the light. Wild-type (left) or *pex7*-1 *pex5*-1 (right) plants were grown for 7 days under white light on medium lacking sucrose. Scale bars represent 1 mm.
Whereas defects in β-oxidation of fatty acids stored in seeds renders many peroxisome-defective mutants sucrose dependent for seedling establishment (Footitt et al., 2002; Fulda et al., 2004; Hayashi et al., 2000; Hayashi et al., 1998; Zolman and Bartel, 2004; Zolman et al., 2001a; Zolman et al., 2001b), the pex7-1 hypocotyl is not markedly shorter than wild type when grown in darkness without added sucrose (Figure 3.9). This lack of sucrose dependence suggests that any defect in peroxisomal β-oxidation of endogenous fatty acids in the pex7-1 mutant may be modest. Adult pex7-1 plants are not discernibly different from wild type in growth rate or morphology (data not shown).

To test whether matrix proteins are imported normally into pex7-1 peroxisomes, I expressed PTS1- and PTS2-tagged versions of GFP in the pex7-1 mutant. As expected, PTS1-tagged GFP (Zolman and Bartel, 2004) is efficiently imported into peroxisomes in pex7-1 (Figure 3.10). The presence of PTS1-GFP in a normal punctate pattern in pex7-1 roots indicates that peroxisome abundance and gross morphology are unaffected by PEX7 deficiency. In contrast, PTS2-tagged GFP is not efficiently imported into pex7-1 peroxisomes; although some faint punctate fluorescence is still observed, PTS2-GFP confers largely diffuse fluorescence similar to cytoplasmically-localized GFP (Figure 3.10). To confirm that this defect observed with an engineered substrate reflected endogenous proteins, I used western blotting to indirectly examine import of 3-ketoacyl-CoA thiolase (thiolase) into peroxisomes. As the PTS2 signal is removed from thiolase following import, the consequent molecular weight shift is diagnostic of peroxisomal import. I detected primarily mature processed thiolase in wild-type seedlings, but some residual unprocessed thiolase remains in pex7-1 seedlings, especially in seedlings grown in the absence of sucrose (Figure 3.11). These results imply that peroxisomal deficiencies observed in pex7-1 result from poor import of PTS2-containing proteins.

Rescue of pex7-1 phenotypes with overexpression of PEX7 revealed that the phenotypes are caused by reduced PEX7 function (Figure 3.9). Because the T-DNA
Figure 3.10. *pex7-1* and *pex5-1* are deficient in PTS2 protein import into peroxisomes.
3-day-old root tips from seedlings grown in 0.1% agar in white light (left panels) and root hair cells from 7-day-old plants grown on PNS (right panels) are shown for wild type, *pex7-1*, and *pex5-1*, and transformants expressing PTS1-GFP, PTS2-GFP, and cytoplasmic GFP. Note the punctate fluorescence in plants expressing PTS1-GFP and wild-type plants expressing PTS2-GFP; fluorescence is diffuse in *pex7-1* and *pex5-1* expressing PTS2-GFP, indicating disrupted PTS2 protein import into peroxisomes. Untransformed plant images were captured at the maximum exposure time used for any of the transformed lines to reveal any autofluorescence. Scale bars represent 200 μm.
Figure 3.11. PTS2 protein import defects in *pex7-1* and *pex5-1*.
A, Thiolase import defects. Precursor thiolase protein is translated with an N-terminal PTS2 sequence that is cleaved after entry into peroxisomes to produce mature thiolase. Protein was extracted from seedlings grown on sucrose and visualized using an anti-thiolase antibody (Kato et al., 1996). B, Thiolase import defects are exaggerated in plants grown without exogenous sucrose. *pex7-1 pex5-1* was omitted because of developmental arrest in the absence of sucrose. C, PEX5 protein is present in all mutants. Protein was extracted from seedlings grown on sucrose and visualized using PEX5 antibody (Zolman and Bartel, 2004). Positions of molecular mass markers (in kDa) are indicated at the left in panels A, B, and C. D, PEX7 message is present in all mutants. *PEX7* mRNA levels relative to an *APRT* control in wild type and each mutant were determined using quantitative real-time reverse-transcription PCR. Error bars represent standard deviations of mean *PEX7* levels expressed in arbitrary units.
insertion in *pex7-1* is upstream of the start codon, it is not obvious whether the mutation causes reduced *PEX7* mRNA accumulation or reduced translation. I examined *PEX7* mRNA accumulation using real-time reverse-transcriptase PCR and determined that any reduction is modest in the mutant (Figure 3.11). Therefore, I ordered rabbit antibodies against two *PEX7* peptides and performed western blots of wild-type, mutant, and *PEX7* overexpression lines. Preliminary western analysis revealed that both antibodies reacted with proteins of ~40 and ~48 kDa (Figure 3.12). The predicted molecular weight of arabidopsis *PEX7* is ~36 kDa. However, it is unlikely that both antibodies cross-react with the same proteins, therefore these bands are likely to contain *PEX7*. Migration at a higher than expected molecular weight may result from extended secondary structure (almost the entire length of *PEX7* is composed of WD40 repeats involved in β-propeller formation (Figure 3.1). One or both of the doublet bands could represent a degradation product of *PEX7*, or a posttranslationally-modified form of *PEX7*. Despite these uncertainties, both bands with both antibodies are fainter in *pex7-1* than in wild type (Figure 3.12), likely indicating reduced *PEX7* protein accumulation in the mutant. Because *PEX7* message was not greatly reduced in *pex7-1* (Figure 3.11), but the presumed *PEX7* protein was (Figure 3.12), the T-DNA may be interfering with mRNA translation more than transcription.

3.B.2. **PTS2-protein import is defective in the PTS1 receptor mutant pex5-1**

Arabidopsis contains a single *PEX5* gene (*At5g56290*; Brickner et al., 1998; Zolman et al., 2000) encoding a protein ~20 and ~28 percent identical to *S. cerevisiae* Pex5p and human PEX5L, respectively (Figure 3.1). *PEX5* proteins contain N-terminal pentapeptide repeat (PPR) domains involved in PEX14 docking at the peroxisome (Nito et al., 2002) and C-terminal tetratricopeptide repeat (TPR) domains necessary for PTS1 protein cargo binding (Gatto et al., 2000). In addition, amongst the TPR repeats is a sequence conserved between human and plant PEX5 proteins and the *PEX7*-binding
Figure 3.12. PEX7 protein accumulation is reduced in pex7-1.
Total protein was extracted from wild-type Col-0 (WT), pex7-1, and progeny of two independent wild-type plants carrying a PEX7 overexpression construct (35SPEX7) after two days of growth in water under continuous white light at 22 °C. Protein was separated by SDS gel electrophoresis, transferred to a membrane, and visualized using α-PEX7 peptide antibodies 105 (A) or 301 (B). Arrowheads mark proteins of equivalent size that appeared in both blots.
domains of the yeast peroxisome docking proteins Pex18p and Pex21p (Einwächter et al., 2001). A fragment of human PEX5L including this region is sufficient for interaction with human PEX7 (Dodt et al., 2001).

The previously-described pex5-1 mutant displays reduced sensitivity to exogenous IBA and a slight growth defect in darkness without exogenous sucrose (Zolman et al., 2000). pex5-1 contains a serine to leucine mutation in the presumptive PEX7-binding region (Figure 3.1). Western blot analysis indicates that the pex5-1 mutant protein accumulates to wild-type levels (Figure 3.11). Intriguingly, the analogous serine is mutated to phenylalanine in a Chinese hamster ovary (CHO) cell line defective in PTS2, but not PTS1, protein import (Matsumura et al., 2000), suggesting that the arabidopsis pex5-1 mutant might similarly have defects in PTS2 import.

To directly examine PTS1 and PTS2 function in the pex5-1 mutant, I observed localization of PTS1- and PTS2-targeted GFP derivatives. Interestingly, I found that pex5-1 is fully competent in PTS1-GFP import (Figure 3.10). In marked contrast, however, PTS2-GFP is not detectably imported into peroxisomes in pex5-1 (Figure 3.10), consistent with the hypothesis that the primary defect in the pex5-1 mutant is in PTS2, rather than PTS1, import. Indeed, PTS2-targeted thiolase is imported less efficiently in pex5-1 than in wild type or pex7-1 (Figure 3.11).

3.B.3. Developmental defects and blocked PTS2-protein import in pex7-1 pex5-1

pex7-1 and pex5-1 were cross-pollinated to produce a pex7-1 pex5-1 double mutant. Like both parents, the double mutant displays reduced sensitivity to exogenous IBA (Figure 3.9). Unlike either single mutant, however, pex7-1 pex5-1 is completely dependent on exogenous sucrose for growth not only in darkness, but also in light (Figure 3.9). Even when provided with sucrose, the double mutant grows more slowly than wild type, as evidenced by reduced root and hypocotyl elongation (Figure 3.9). Moreover, the possible lateral root defects observed in the single mutants are exacerbated in the double
mutant; 9-day-old pex7-1 pex5-1 seedlings lack lateral roots (Figure 3.9). Consistent with these morphological defects, 3-day-old double mutant seedlings apparently lack processed thiolase (Figure 3.11), suggesting a severe block in import of thiolase, and presumably other PTS2 proteins, into peroxisomes.

In addition to these seedling defects, adult pex7-1 pex5-1 plants are dramatically less robust and less fecund than wild type or either parent (Figure 3.13). Examination of siliques of different ages reveals apparently normal fertilization indicated by the presence of plump developing seeds in green siliques, but defects become increasingly apparent during the course of seed maturation (Figure 3.13). A majority of mature double mutant seeds are shrunken (Figure 3.13). Seed development is dependent upon PEX7 dosage; plants homozygous for pex5-1 and heterozygous for pex7-1 generate an intermediate number of shrunken seeds (Figure 3.13). In contrast, pex5-1 is fully recessive for seed morphology in the pex7-1 background, as pex7-1/pex7-1 PEX5/pex5-1 plants produce normal seeds (Figure 3.13). Among pex7-1 pex5-1 seeds with a wild-type appearance, fewer than half germinate when grown on media supplemented with sucrose, whereas only occasional abnormally-shaped seeds germinate (Figure 3.13).

Among pex7-1 pex5-1 mutant seeds that germinate, abnormalities in seedling development become apparent (Figure 3.14). About 15% of mutant seedlings exhibit various degrees of cotyledon fusion (Figure 3.14), whereas such aberrant seedling morphology is seen in fewer than 1.2% of wild type or either single mutant (n ≥ 112, data not shown). Most pex7-1 pex5-1 seedlings with fused cotyledons develop into adult plants resembling siblings with unfused cotyledons, though a small percentage arrest and perish as seedlings (data not shown). These results imply that PTS2-dependent protein import into peroxisomes is necessary for normal embryo development.
Figure 3.13. **pex7-1 pex5-1 double mutant phenotypes.**
A, Seed development is aberrant in pex7-1 pex5-1. One valve was removed from wild-type and pex7-1 pex5-1 siliques to reveal developing seeds of increasing age from left to right. Scale bar represents 1 mm. B, Mature pex7-1 pex5-1 seeds are shrunken. Scale bar represents 1 mm. C, pex7-1 pex5-1 adult plants have reduced stature. Plants were grown under white light on PNS for 14 days, then transferred to soil and grown an additional 17 days. Scale bar represents 1 cm. D, Percent seeds with normal filled morphology. Mature seeds were assayed for plump appearance. Bars represent means + standard deviations from progeny of three plants of the indicated genotype; n ≥ 60 seeds per plant. E, Percent germination in single and double mutants. pex7-1 pex5-1 were sorted by seed morphology in D. Seedlings were grown on medium supplemented with 45 mM sucrose and assayed for germination (radicle emergence) after 9 days. Bars represent means + standard deviations (or standard error of measurement for pex5-1) from progeny of three plants of the indicated genotype (progeny of two pex5-1 plants); n ≥ 23 seeds per plant, except pex7-1 pex5-1 filled seeds where n ≥ 10 seeds per plant. Asterisks indicate values significantly different from wild type (P < 0.02; one-tailed t-test assuming unequal variance).
Figure 3.14. Cotyledon fusion in the *pex7-I pex5-I* double mutant.

*pex7-I pex5-I* plants were grown for 7 days on sucrose-supplemented medium and frequencies of plants with wild-type (A), asymmetric (B), partially fused (C), and fused (D) cotyledons were determined. Ratios below B, C, and D represent fraction of *pex7-I pex5-I* with the indicated degree of fusion among progeny of three double mutant plants. Scale bar represents 1 mm.
3.B.4. Attempted rescue of pex5-1 with PEX7-SKL

Mislocalization of PTS2 proteins in pex5-1, though PTS1-GFP localization is normal, indicate a PTS2-specific deficiency (Figures 3.10 and 3.11). Because PTS2 protein import is incompletely blocked in pex5-1 (Figure 3.11), it is possible that overexpression of PEX7 could partially restore peroxisomal function in pex5-1. Therefore, I transformed pex5-1 with a PEX7 overexpression construct and assayed IBA sensitivity in the resultant T2 progeny (Figure 3.15); if the introduced gene could restore IBA response, 3/4 of the T2 progeny should be more inhibited by IBA than the parental line. PEX7 overexpression was not sufficient to restore IBA response in pex5-1 (Figure 3.15). However, this does not disprove the hypothesis that the pex5-1 lesion interferes with PEX7 binding to yield reduced PTS2 protein import and IBA response. It remains possible that PEX7 does not accumulate to significantly higher levels in these lines. In addition, PEX7 availability for binding may be limited, for example, by PTS2 protein levels if only the cargo-bound form of PEX7 interacts with PEX5. Similarly, overexpression of PEX5 did not restore IBA sensitivity to pex7-1 (Figure 3.15), indicating that, if PEX5 is overexpressed in these lines, PEX5 levels are not limiting for PEX7 function. Western blot analysis could be employed to test whether the proteins accumulate as expected in these lines.

An alternate approach could be employed to test the hypothesis that the pex5-1 phenotypes result from reduced interaction with PEX7 in vivo. Because oligomeric proteins are imported into peroxisomes (Kato et al., 1999; McNew and Goodman, 1994), it may be possible to rescue pex5-1 by adding a PTS1 signal sequence to the PTS2 receptor PEX7, thus demonstrating that the mutant phenotypes result from PTS2 defects.

I added codons for a PTS1 signal sequence to the PEX7 gene using PCR with a modified 3’ oligo inserting lysine and leucine codons between the terminal serine and stop codons of genomic PEX7. I created amplification products of wild-type PEX7 and PTS1-PEX7 under control of the endogenous PEX7 promoter. PEX7 and PTS1-PEX7
Figure 3.15. Coordinate overexpression.
Wild type, pex7-1, and pex5-1 lines carrying 35S promoter-driven overexpression constructs of PEX7 and PEX5 were assayed for IBA resistance. Plants were grown for eight days on PNS or PNS supplemented with 5 μM IBA under yellow light filters at 22 °C. Bars represent the number of individuals within the population with the indicated root length. F2 progeny of several transformants were included in each trial.
PCR products will be subcloned into a shuttle vector and subsequently into the pBENEblue (LeClere et al., 2004) plant transformation vector. The constructs will be transformed into wild type, *pex7-1*, *pex5-1*, and *pex7-1 pex5-1*. Homozygous transformed T3 lines will be identified on the basis of BASTA resistance as above. These lines will be compared to untransformed plants to assay for rescue of known phenotypes for each mutant.

### 3.B.5. PEX7 TILLING

Because the sole allele of *pex7* examined in this study is a partial knockdown (Figures 3.11 and 3.12), and because combining *pex7-1* with the *pex5-1* mutation that partially compromises the same pathway enhances the *pex7-1* mutant phenotype, it is likely that abolishing PEX7 function entirely will result in a more severe phenotype than that observed in *pex7-1*. For this reason, I utilized the reverse-genetic TILLING facility to identify additional alleles with mutations in the *PEX7* gene. TILLING uses a mismatch cleavage technique to identify plants with EMS-induced mutations within a region of interest (Till et al., 2003). The Arabidopsis TILLING Project (McCallum et al., 2000) identified five *pex7* alleles, none of which are early stop codons that might be expected to fully compromise PEX7 function (Figure 3.1). It remains possible that some of the missense mutations identified through TILLING will cause more severe phenotypes than *pex7-1* and thereby reveal important residues within the protein as well as reinforce the general importance of PEX7 and the PTS2 pathway for plant development.
Chapter 4: Additional genes involved in IBA response and embryogenesis

4.A. Peroxisome biogenesis mutants

Peroxisomes are small organelles that house many cellular processes, including fatty acid β-oxidation, a process that takes place in mammalian mitochondria and peroxisomes, but is considered to be solely peroxisomal in plants and yeast (Gerhardt, 1992; Kindl, 1993). Many proteins in yeast and mammals have been identified as essential for peroxisome biogenesis and named PEROXIN (PEX) proteins (Distel et al., 1996). While at least 26 PEX genes have been described, only six plant peroxin mutants have been previously characterized: arabidopsis pex5-1 (Zolman et al., 2000), pex14-1/ped2-1 (Hayashi et al., 2000), pex16-1/sse1-1 (Lin et al., 1999), and pex2-1/te3d-1 (Hu et al., 2002), pex10 (Schumann et al., 2003; Sparkes et al., 2003), and pex6-1 (Zolman and Bartel, 2004). We have begun to identify and characterize arabidopsis mutants in other PEX genes identified through sequence homology. Other groups have attempted reverse genetics with putative peroxisome biogenesis genes in arabidopsis using RNAi knockdown (Hayashi et al., 2005). Though such lines may help reveal that the targeted genes are indeed involved in peroxisome biogenesis, genetic knockouts remain necessary for fully understanding the role of the genes in plant development. RNAi knockdown often causes only partial loss of function, is often troubled by nonspecific side effects, and may not consistently affect expression through different developmental stages.

PEX5 is the receptor for the majority of cytosolic proteins bound for the peroxisome, recognizing a C-terminal Peroxisomal Targeting Signal Type 1 (PTS1) sequence with the consensus SKL (Neuberger et al., 2003). Once cargo is bound (Gouveia et al., 2003), the PEX5 complex docks with factors on the peroxisomal membrane via a PEX13-PEX14-PEX17 complex, and then is translocated into the
peroxisome, followed by recycling of PEX5 to the cytosol (Dammai and Subramani, 2001). Overexpression of PEX5 in yeast rescues mutants defective in certain other peroxins such as pex4 (van der Klei et al., 1998) and pexl4 (Salomons et al., 2000), emphasizing the central role of PEX5 in peroxisomal function.

PEX7 is the other known receptor for cytosolic proteins bound for the peroxisome (Osumi et al., 1991; Swinkels et al., 1991). Tobacco PEX7 recognizes an N-terminal sequence with the Peroxisomal Targeting Signal 2 (PTS2) consensus -R/K-X_5-H/Q-A/L/F- (Flynn et al., 1998). With PTS2-containing cargo bound, the PEX7 complex translocates into the peroxisome (Mukai et al., 2002). Mammalian PEX7 requires interaction with PEX5 to import PTS2 proteins into the peroxisome (Otera et al., 2000). No evidence supports PEX5-PEX7 interaction in yeast. A single PEX7 gene is found in arabidopsis (Schumann et al., 1999); the encoded protein is ~59% and ~40% identical to the human and yeast homologs, respectively. Arabidopsis PEX7 more closely resembles the PEX5-binding mammalian isoform of PEX7 (Olsen, 1998), and arabidopsis PEX5 and PEX7 interact in a two-hybrid assay (Nito et al., 2002). Indeed, PEX7 is necessary for peroxisome function in arabidopsis and depends on PEX5 for delivery of PTS2 cargo to the peroxisome (Chapter 3); thus, plant PTS2 proteins rely on PEX5, and thereby the same docking and translocation factors required for PTS1 proteins.

PEX13 in mammals and yeast is a transmembrane protein involved in peroxisomal protein import (Erdmann and Blobel, 1996). PEX13 bears a C-terminal Src Homology 3 (SH3) domain that faces the cytosol and binds the PTS1 receptor PEX5 (Urquhart et al., 2000). In addition, PEX13 interacts with the PTS2 receptor PEX7, though the interaction may not be direct (Girzalsky et al., 1999). Generally, PEX13 is thought to form a complex with PEX14 and PEX17 necessary for PEX5 and PEX7 docking to the cytosolic face of the peroxisomal membrane (Huhse et al., 1998). Arabidopsis PEX13 has not yet been functionally identified (Nito et al., 2002), but two homologs to yeast and mammalian PEX13 are present. One (At3g07560) is ~15%
identical to yeast PEX13, but is only ~11.5% identical to human PEX13 and lacks any predicted transmembrane domain (Figure 4.1). The other (At4g21900; Figure 4.2) is ~16% and ~12% identical to human and yeast PEX13, respectively, though much of the homology is in the SH3 domain; it bears nine predicted transmembrane domains.

PEX17 is a small protein associated with the PEX13 peroxisomal docking complex (Huhse et al., 1998; Smith et al., 1997; Snyder et al., 1999). While the role of PEX17 in peroxisomal matrix protein import is poorly understood, pex17 mutants in other organisms have peroxisome biogenesis defects (Huhse et al., 1998; Smith et al., 1997; Snyder et al., 1999). A single PEX17-like gene (At4g18200) has been reported. However, examination of cDNAs within the locus reveals three independent PEX17-like genes in tandem (renamed At4g18195, At4g18197, and At4g18205; Figure 4.3). In addition, the unlinked gene At5g62970 appears to encode a PEX17-like protein (Figure 4.3). To date, no plant pex17 mutants have been described.

PEX2, PEX10, and PEX12 are integral peroxisomal membrane proteins with zinc RING finger domains. Mutants deficient in these proteins are defective in peroxisomal protein import, and mammalian PEX12 interacts with PEX5, the PTS1 receptor (Okumoto et al., 2000). In addition, the three proteins appear to be a complex: in mammals, PEX12 interacts with PEX10, and PEX10 interacts with PEX2 (Okumoto et al., 2000). A complex of PEX2, 10, and 12 therefore is thought to assist in the translocation of peroxisomal targeting signal receptors and their cargo. Though structurally similar, these proteins bear only ~10% identity to each other in arabidopsis (Figure 4.4). The zinc RING finger domain is found in E3 ubiquitin ligases, and it has been suggested that all RING finger proteins act as E3s (Freemont, 2000). Whereas ubiquitination is most familiar as a mechanism of targeting substrates for degradation, it also can play roles in intracellular sorting of certain proteins (Katzmann et al., 2001). Further, S. cerevisiae Pex5p is ubiquitinated in a process dependent on Pex10p (Platta et al., 2004). In plants, PEX10 is an essential gene. Transposon-induced disruption of
Figure 4.1. An arabidopsis PEX13-like protein.

PEX13 protein sequences from arabidopsis (At3g07560), human (Hs), mouse (Mm), roundworm (Ce), budding yeast (Sc), and the yeast *Pichia pastoris* (Pp) were aligned using the MegAlign program ClustalW algorithm (DNASTAR, Madison, WI). Residues shaded black are identical in three or more sequences; residues shaded grey are chemically similar in three or more sequences. The T-DNA location upstream of the PEX13 translational start codon is indicated with a black triangle (see Table 4.1 for details). Black line indicates a transmembrane domain in human PEX13; grey line indicates an SH3 domain.
Figure 4.2. A possible alternative arabidopsis PEX13.
C-terminal ends of At4g21900 (PEX13B) and PEX13 proteins from human (Hs) and mouse (Mm) were aligned using the MegAlign program ClustalW algorithm (DNASTAR, Madison, WI). Residues shaded black are identical in all sequences; residues shaded grey are chemically similar in all sequences. Grey line indicates an SH3 domain.
Figure 4.3. Arabidopsis PEX17-like proteins.
Arabidopsis (At) and the fungi Yarrowia lipolytica (Yl), Pichia pastoris (Pp), and Saccharomyces cerevisiae (Sc) were aligned using the MegAlign program ClustalW algorithm (DNASTAR, Madison, WI). Residues shaded black are identical in a majority of sequences; residues shaded grey are chemically similar in a majority of sequences. T-DNA insertion locations are indicated with black triangles (see Table 4.1 for details), missense mutations are indicated with arrows and replacement amino acid symbols. Note that the similar names of the first three proteins indicate adjacent genes.
**Figure 4.4.** Arabidopsis PEX2, PEX10, and PEX12.

Arabidopsis proteins were aligned using the MegAlign program ClustalW algorithm (DNAStar, Madison, WI). Residues shaded black are identical in a majority of sequences; residues shaded grey are chemically similar in a majority of sequences. Plus signs indicate metal-coordinating residues of RING finger domains.
*PEX10* results in embryonic arrest (Schumann et al., 2003; Sparkes et al., 2003), and insertional disruption of *PEX2* also confers embryo lethality (Hu et al., 2002). Interestingly, a missense mutation of arabidopsis *PEX2, ted3-1*, results in suppression of *det1-1*, a de- etiolated mutant that develops as if in light when grown in darkness (Hu et al., 2002). Thus, plant peroxisomes in general, and specifically arabidopsis PEX2, may play a role in photomorphogenesis. Plant mutants of *PEX12* have not been described.

PEX19 is a membrane-associated protein required for early events in peroxisome biogenesis in some organisms (Fransen et al., 2001; Götte et al., 1998). Peroxisomal membranes, identified by the presence of other peroxisomal membrane proteins, do not form in yeast *pex19* mutants (Götte et al., 1998). Human and yeast PEX19 bear a C-terminal prenylation sequence (CAAX-box motif) that is the site of modification by an aliphatic prenyl terpenoid molecule that may facilitate association of PEX19 with peroxisomal integral membrane proteins (Fransen et al., 2001). A single arabidopsis *PEX19*-like gene has been identified previously (*At3g03490*). However, I performed a BLAST search and identified another *PEX19*-like gene in arabidopsis, *At5g17550* (*PEX19B*); both PEX19 and PEX19B bear the C-terminal prenylation attachment sequence (Figure 4.5).

PEX11 regulates peroxisome abundance. Overexpression of *PEX11* in human cells and yeast causes an increase in peroxisome number, and mouse cells lacking *PEX11* have fewer peroxisomes (Li and Gould, 2002). Recent evidence suggests that PEX11 allows division of existing peroxisomes through indirect recruitment of the Dynamin-like GTPase DLP1 (Li and Gould, 2003). Direct interaction of PEX11 with coatamer proteins has been observed, but mutations that affect coatamer binding do not affect *in vivo* PEX11 function (Maier et al., 2000). Humans possess two PEX11 isoforms, while yeast have only a single PEX11 (Abe and Fujiki, 1998), and arabidopsis harbors as many as five PEX11 homologs ranging from ~11% to ~22% identical to the human PEX11α (Figure 4.6). No plant *pex11* mutants have been described.
**Figure 4.5. Arabidopsis PEX19-like proteins.**

PEX19 proteins from arabidopsis (At), human (Hs), budding yeast (Sc), the fungus *Pichia pastoris* (Pp), and the fungus *Pichia angusta* (Pa) were aligned using the MegAlign program ClustalW algorithm (DNASTAR, Madison, WI). Residues shaded black are identical in all sequences; residues shaded grey are chemically similar in all sequences. The locations of T-DNA disruprtional insertions are indicated with triangles (see Table 4.1 for details); a black bar at the C-terminus indicates a putative prenylation modification CAAX box site.
Figure 4.6. Arabidopsis PEX11 proteins.

Putative arabidopsis (At) PEX11 proteins, two human (Hs) isoforms, and a budding yeast (Sc) PEX11 were aligned using the MegAlign program ClustalW algorithm (DNASTAR, Madison, WI). Residues shaded black are identical in a majority of sequences; residues shaded grey are chemically similar in a majority of sequences. The location of a T-DNA insertion upstream of At1g47750 is indicated with a triangle (see Table 4.1 for details).
Characterization of arabidopsis mutants defective in putative peroxisome biogenesis genes will confirm or revoke the role of each in peroxisome biogenesis. In addition, characterization of partial and complete loss-of-function alleles of mutants defective in particular aspects of peroxisome biogenesis will reveal the roles for peroxisomes and peroxisomal processes in plant development. I have therefore undertaken a reverse genetic analysis of several arabidopsis PEX-like genes.

4.A.1. Peroxisomal docking proteins


Homozygous pex13-1 plants were initially found to be less sensitive than wild type to IBA (Figure 4.7). However, after backcrossing to the parental line and selecting for homozygosity by PCR amplification of mutant DNA, IBA resistance was no longer observed (Figure 4.8), indicating the presence of an unlinked locus that affected IBA sensitivity in the parental line. The T-DNA insertion in pex13-1 is upstream of the gene, so that this allele may cause a knockdown of PEX13 expression rather than a knockout, similar to the case in pex7-1 (Chapter 3). Supporting this hypothesis, I have been unable to obtain a homozygous line with a second pex13 allele bearing a T-DNA within the coding region, consistent with embryo lethality (data not shown). Therefore, PEX13 may be an essential gene, but the pex13-1 allele may not sufficiently compromise PEX13 activity to confer an observable phenotype.

A second potential arabidopsis PEX13 (PEX13B) bears identity mainly with the SH3 domain of other PEX13 proteins (Figure 4.2). T-DNA disruption alleles of this gene have been procured but not characterized (Table 4.1). Characterization of these alleles may be important because PEX13B is a putative transmembrane protein. PEX13 in other organisms is localized on the peroxisomal membrane (Erdmann and Blobel, 1996);
Figure 4.7. *pex13-1* initially appeared IBA-resistant.
Seedlings were grown for eight days under yellow light filters at 22 °C on PNS or PNS supplemented with 5 μM IBA. Bars represent mean root length versus root length for each genotype on unsupplemented medium + standard deviations; *n* = 12. Vertical line separates individual experiments. *pex7-1* is shown for comparison.
Figure 4.8. IBA and 2,4-DB response in *pex2*, *pex13*, and *pex19* mutants. Seedlings were grown for seven days under yellow light filters at 22 °C on PNS supplemented with IBA or 2,4-DB. Points represent mean root length +/- standard deviations; *n* ≥ 4. *pex7-1* is shown for comparison.
Table 4.1. T-DNA insertional disruption *pex* mutants.

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*(Alonso et al., 2003)*

*None indicates failed PCR reaction attempts; nd indicates untested reactions.

*Primer sequences are listed in Table 2.1.*

*Locations written in black are sequence-verified; those in grey are not.*

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however, the only previously-identified PEX13 (At3g07560) in Arabidopsis has no predicted transmembrane domains.

4.A.1.B. pex17

In fungi, PEX17 is a necessary component of the peroxisome docking complex (Huhse et al., 1998; Smith et al., 1997). Originally, a single, three-exon gene was identified as PEX17 in Arabidopsis; however, I examined translated protein sequence and cDNAs and found that the locus is instead three tandemly-repeated PEX17-like genes. I obtained a putative PEX17-disrupted Arabidopsis line carrying a T-DNA in gene At4g18195 (Figure 4.3). Because the PEX17 tandem repeat region is small, the T-DNA may disrupt expression of one or more version of PEX17. Regardless, the insertion in heterozygous pex17a-1 does not confer IBA resistance (Figure 4.9). However, I was unable to obtain homozygous lines, consistent with the possibility that the mutation is homozygous inviable. I obtained TILLING lines with missense mutations in a PEX17-like gene (At4g18205; Figure 4.3). Two of these lines have been characterized before backcrossing and do not appear to confer reduced sensitivity to IBA, though slight 2,4-DB resistance in pex17c-9 will require backcrossing and further characterization (Figure 4.10).

4.A.2. RING finger peroxins

PEX2, PEX10, and PEX12 are thought to function in docking or import of PEX5, and thereby function in import of proteins to the peroxisomal matrix (Okumoto et al., 2000). These RING finger proteins may act as E3 ubiquitin ligases.

I have isolated populations heterozygous for T-DNA disruptions within the coding sequence of PEX2 and PEX12 (Figures 4.11 and 4.12; Table 4.1). Heterozygous pex2-2 and pex12-1 mutants retain wild-type sensitivity to IBA (data not shown). Unable to isolate individuals homozygous for either mutation, I examined mature siliques from
Figure 4.9. *pex17a-1* is sensitive to IBA.
Seedlings were grown for seven days under yellow light filters at 22 °C on PNS or PNS supplemented with IBA. Bars represent mean root length + standard deviations; *n* ≥ 13.
Figure 4.10. IBA and 2,4-DB response in pex2, pex12, and pex17 mutants. Seedlings were grown for seven days under yellow light filters at 22 °C on PNS supplemented with IBA or 2,4-DB. Points represent mean root length +/- standard deviations; n ≥ 4.
Figure 4.11. PEX2 proteins.

PEX2 proteins from arabidopsis (At), human (Hs), mouse (Mm), rat (Rn), budding yeast (Sc), the fungus *Pichia pastoris* (Pp), and the protist *Trypanosoma brucei* (Tb) were aligned using the MegAlign program ClustalW algorithm (DNASTAR, Madison, WI). Residues shaded black are identical in a majority of sequences; residues shaded grey are chemically similar in a majority of sequences. The location of a T-DNA insertion is indicated with a triangle (see Table 4.1 for details). Missense mutations are indicated with arrows and the replacement amino acid or an asterisk for a stop codon (see Table 4.2 for details). Plus signs indicate metal coordinating residues in the RING finger domain.
Figure 4.12. PEX12 proteins.

PEX12 proteins from the dicotyledonous plant Arabidopsis (At), the monocotyledonous plant rice (Os), human (Hs), rat (Rn), C. elegans (Ce), budding yeast (Sc), and the fungus Pichia pastoris (Pp) were aligned using the MegAlign program ClustalW algorithm (DNASTAR, Madison, WI). Residues shaded black are identical in a majority of sequences; residues shaded grey are chemically similar in a majority of sequences. The location of a T-DNA insertion is indicated with a triangle (see Table 4.1 for details). Missense mutations are indicated with arrows and the replacement amino acid or an asterisk for a stop codon (see Table 4.2 for details). Plus signs indicate metal coordinating residues in the RING finger domain.
heterozygous and wild-type siblings (Figures 4.13 and 4.14). Results indicate that both pex2-2 and pex12-1 mutations are lethal when homozygous. Previous research has shown that T-DNA disruption of PEX2 confers embryo lethality and that heterozygous pex2 seed pods are shorter as a result (Hu et al., 2002). However, when I examined seed pod length in pex2 and pex12, I found no significant differences from wild type (Figure 4.13). However, examination of mature seed pods revealed shriveled seeds similar to those seen in pex7-1 pex5-1 (Figure 4.13; see Chapter 3 for discussion of pex7-1 pex5-1), indicative of aborted development at a later embryonic stage than would be indicated by shorter seed pods.

I was unable to isolate a pex10 T-DNA allele; previous studies of PEX10 disruption reveal that it is an essential gene (Schumann et al., 2003; Sparkes et al., 2003), and T-DNA insertions in pex2 and pex12 are lethal. Therefore, I sought to obtain mutations in these genes causing less severe phenotypes. Utilizing the TILLING facility, which can recover point mutations in genes of interest (Colbert et al., 2001), I isolated five additional pex2 alleles (Figure 4.11; Table 4.2), eight additional pex12 alleles (Figure 4.12; Table 4.2), and four pex10 alleles (Figure 4.15). A subset of TILLING alleles for the RING finger peroxins have been phenotypically characterized, and none displays a peroxisomal defective phenotype (Figure 4.10). However, I found that the previously-described pex2 mutant ted3, isolated as a suppressor of the de-etioloated phenotype of det1 (Hu et al., 2002), displays previously undescribed 2,4-DB resistance (Figure 4.8). In addition, the pex2-5 allele appears to have reduced sensitivity to 2,4-DB (Figure 4.9). These are the first data that directly reveal a role for PEX2 in plant peroxisomal function.

In addition to further characterization of TILLING alleles of RING finger peroxins, the embryo lethality phenotype of insertional disruption alleles warrants further examination. The reason for peroxisome biogenesis gene knockout lethality in plants, though previously observed, remains unknown. Because pex10 knockouts arrest at early
Figure 4.13. Insertional disruption of PEX2 may cause embryonic arrest.

Four mature siliques (seed pods) at positions 5-8 from the terminal siliques were measured (A), opened, and seeds were visually assayed for plump normal appearance ("filled seeds", B) or shriveled, abnormal appearance ("shrunken seeds", C). At least four inflorescences from multiple pex2-1 heterozygous plants were examined. Bars represent means ± standard deviations; n ≥ 37.
Figure 4.14. Insertional disruption of *PEX12* may cause embryonic arrest.
Four mature siliques (seed pods) at positions 5-8 from the terminal siliques were measured (A), opened, and seeds were visually assayed for plump normal appearance ("filled seeds", B) or shriveled, abnormal appearance ("shrunken seeds", C). At least four inflorescences from multiple *pex12-1* heterozygous plants were examined. Bars represent means ± standard deviations; *n* ≥ 16.
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*aNumbers denote location in reference to initial A of start codon in mature mRNA sequence.*

*bNumbers denote location in reference to initial methionine; * indicates a stop codon.*

*Text is missing.*

`Check mark indicates that a homozygous line has been obtained.`

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Figure 4.15. PEX10 proteins.

PEX10 proteins from the monocotyledonous plant arabidopsis (At), the monocotyledonous plant rice (Os), human (Hs), fly (Dm), budding yeast (Sc), the fungus Pichia pastoris (Pp), and the fungus Yarrowia lipotitica (Yl) were aligned using the MegAlign program ClustalW algorithm (DNASTAR, Madison, WI). Residues shaded black are identical in a majority of sequences; residues shaded grey are chemically similar in a majority of sequences. The location of a T-DNA insertion is indicated with a triangle (see Table 4.1 for details). Missense mutations are indicated with arrows and the replacement amino acid. Plus signs indicate metal coordinating residues in the RING finger domain.
stages of embryogenesis (Schumann et al., 2003; Sparkes et al., 2003), it will be interesting to determine when knockout alleles of pex2 and pex12 arrest.

4.A.3. Peroxisome structural biogenesis factors

4.A.3.A. pex19-1 and pex19B-1

PEX19 is a protein required for early steps of peroxisome biogenesis in both mammals and fungi (Fransen et al., 2001; Snyder et al., 1999). I have identified two potential PEX19 genes in arabidopsis and isolated lines harboring T-DNA insertional disruptions near each gene (Figure 4.5). No phenotypes have been observed in lines carrying pex19-1 or pex19b-1 T-DNAs (Figure 4.8); however, PCR difficulties have made determination of the genotypes of these lines difficult to ascertain. Because phenotypes were not observed in either mutant line, either PEX19 gene may be able to compensate for loss of the other. Therefore, we have crossed pex19-1 and pex19b-1 and have F2 progeny of the cross that remain to be characterized. We hypothesize that loss of both copies of PEX19 will result in embryo lethality similar to that seen in loss-of-function alleles of many other arabidopsis peroxisome biogenesis factors. It may also be possible that a plant having only one functional copy of one PEX19 gene will have an intermediate phenotype such as reduced IBA sensitivity.

4.A.3.B. FPS1 is not necessary for peroxisome function

As in other organisms, both arabidopsis PEX19 and PEX19B bear C-terminal prenylation motifs (Figure 4.5). The prenyl moiety is a farnesyl pyrophosphate-derived molecule and is important for PEX19 function (Götte et al., 1998). Hypothesizing that potentially deficient farnesyl pyrophosphate levels in a farnesyl pyrophosphate synthase fps1-1 mutant (SALK_004298; Dereth Philips, unpublished data) might compromise PEX19 prenylation and thereby affect PEX19 activity, we tested fps1-1 for peroxisome
defective phenotypes. The mutant was not defective in IBA or 2,4-DB response (Figure 4.16), failing to reveal a connection between FPS1 and peroxisome biogenesis. However, an additional FPS gene is present in Arabidopsis, so it remains possible that this pathway is important for peroxisome biogenesis. Analysis of future doubly-compromised fps mutants (if such mutants are viable) will allow the testing of this hypothesis.

4.A.4. The peroxisome proliferation factor PEX11

A single Arabidopsis gene was first identified as PEX11 (At1g47750). However, genome-wide analysis revealed the presence of five putative PEX11 genes (Figure 4.6). I isolated an Arabidopsis lineage carrying a T-DNA disruption within one PEX11 gene (At1g47750; Figure 4.6; Table 4.1). Plants homozygous for pex11-1 were initially found to have slightly reduced sensitivity to IBA (Figure 4.7). However, like pex13-1, pex11-1 displayed wild-type sensitivity to IBA and 2,4-DB after backcrossing to Col-0 (Figure 4.17), indicating the presence of an unlinked locus that affected IBA sensitivity. Because I have been unable to identify a phenotype in backcrossed pex11-1, multiple mutations in various PEX11 genes may be necessary to reveal the contribution of PEX11 to peroxisome biogenesis and plant development.

4.B. Forward genetics

In addition to reverse genetic studies of putative plant peroxisome biogenesis genes, I characterized several mutants previously isolated by Bethany Zolman and Melanie Monroe-Augustus derived in an unbiased forward genetic mutant screen of EMS-mutagenized plants selected for reduced sensitivity to root elongation inhibition by IBA. The IBA response mutant screen has identified both Arabidopsis peroxins and other peroxisomal proteins that influence IBA metabolism through peroxisome biogenesis or more directly (Zolman and Bartel, 2004; Zolman et al., 2001a; Zolman et al., 2001b; Zolman et al., 2000).
Figure 4.16. *farnesy1 pyrophosphate synthase 1* responds to IBA.
Seedlings were grown for eight days under yellow light filters at 22 °C on PNS or PNS supplemented with IBA. Bars represent mean root length + standard deviations; *n* ≥ 10. *pex7-1* is shown for comparison.
Figure 4.17. *pex11-1* responds normally to IBA. Seedlings were grown for eight days under yellow light filters at 22 °C on PNS or PNS supplemented with IBA. Bars represent mean root length ± standard deviations; \( n \geq 9 \).
4.B.1. B884 is chy1-6

The mutant B884 is defective in specific peroxisomal processes. The mutant is resistant to root elongation inhibition by IBA and 2,4-D, but sensitive to IAA and 2,4-D (Figure 4.18 and data not shown), indicating a defect in β-oxidation. I utilized recombination mapping of the recessive reduced IBA sensitivity in B884 to localize the mutation near the molecular marker MBK5 on Arabidopsis chromosome 5. This region is near a candidate gene, CHY1, mutations in which confer similar phenotypes to B884 and have frequently emerged from IBA response mutant screens (Zolman et al., 2001a). Sequencing the CHY1 gene in B884 revealed a single G to A mutation at position 916 (where 1 is the A of the translational initiation codon), consistent with the EMS mutagen employed in the mutant screen. This is the last residue of exon seven, and would likely disrupt a splicing site, resulting in translation of an intron and a premature truncation of the CHY1 protein. We therefore renamed this mutant chy1-6. The mutant is compromised in a peroxisomal β-hydroxyisobutyryl-CoA hydrolase involved in valine catabolism; accumulation of a toxic intermediate is thought to negatively impact peroxisomal processes including β-oxidation (Lange et al., 2004; Zolman et al., 2001a).

4.B.2. B491

The putative mutant B491 is also resistant to IBA and 2,4-DB, but sensitive to IAA and 2,4-D (Figure 4.18). B491 makes wild-type numbers of lateral roots and responds to induction of lateral root proliferation by IBA (data not shown). IBA resistance in B491 has a complex inheritance pattern (data not shown) that rendered the mutation difficult to map. Perhaps a strategy such as reverse mapping (picking F2 plants with wild-type IBA responses) will allow for the identification of a region containing the relevant mutation, and, ultimately, the gene defective in B491.
Figure 4.18. Auxin responses in B292, B491, and B884.
Seedlings were grown for eight days under yellow light filters at 22 °C on PNS or PNS supplemented with the indicated synthetic auxin. Note that B292, B491, and B884 are less sensitive than wild type to the 2,4-D precursor 2,4-DB (A), but B292 and B884 respond normally to 2,4-D itself (B). Bars represent mean root length + standard deviations; points represent mean root length +/- standard deviations; n ≥ 6.
4.B.3. B292

Like B884 and B491, the putative mutant B292 is resistant to proto-auxins that must be β-oxidized for activation (Figure 4.18). Also like B491, B292 makes wild-type numbers of lateral roots and responds to IBA induction of lateral root proliferation (data not shown). As with B491, reduced IBA sensitivity in B292 is partially dominant (data not shown), requiring painstaking care in recombination mapping studies. Jean Bao and Erin Woodward tested the progeny of mapping plants to identify homozygous mutant lines, then characterized molecular markers in these mutants to localize a region on Arabidopsis chromosome 5 near the molecular marker GA3 linked to IBA resistance in B292. However, overlap between heterozygous regions in mapping plants precluded identification of a region that must contain the mutation responsible for B292 phenotypes. Perhaps more exhaustive characterization of mapping plant lines will allow reclassification of some of the mapping plants heterozygous across the region of interest, thereby enabling the search for the gene defective in B292.

4.B.3.A. B292 is not iaa28

Despite the difficulties in mapping B292, I scanned the region on chromosome 5 linked to the mutant phenotypes for genes that could cause the mutant phenotypes. The Aux/IAA gene IAA28 is in this region, but is an unlikely suspect, because mutation in iaa28-1 that stabilizes the encoded protein confers resistance to IAA as well as greatly reduced lateral root proliferation (Rogg et al., 2001). However, any phenotype in a loss-of-function iaa28 allele remains unidentified. Because IAA28 is linked to auxin response, it was fully sequenced by Erin Woodward and no mutations were identified. Therefore, the molecular basis for B292 phenotypes remains unresolved.
Chapter 5: IBA and IAA regulate distinct but largely overlapping sets of genes

5.A. IBA effects and experimental design

Though a preponderance of historical research has focused on the natural auxin IAA, the commercially-utilized auxin indole-3-butyric acid has recently been shown to exist naturally in plants (Ludwig-Müller et al., 1993). IBA differs from IAA only by the presence of two additional methylene units in the carboxyl side chain (Figure 1.3). Genetic evidence suggests that IBA acts, at least in part, through conversion to IAA via a peroxisomal process similar to fatty acid β-oxidation (Zolman et al., 2000). For example, the arabidopsis mutant pxa1-l, defective in a peroxisomal membrane fatty-acyl CoA transporter, is less sensitive than wild type to exogenous IBA (Zolman et al., 2000). The defects in a transporter suggest that pxa1-l is deficient in import of IBA into peroxisomes for β-oxidation (Zolman et al., 2000). However, the mutation in pxa1-l results in a truncation very near the C-terminus, and may represent only a partial loss-of-function allele (Zolman et al., 2000). Indeed, pxa1-l, though less sensitive to IBA than wild type, is not IBA-insensitive; the mutant responds to high levels of IBA.

Not only IBA metabolism is affected in pxa1-l. Loss of PXA1 function also causes defective fatty acid β-oxidation (Footitt et al., 2002). Because β-oxidation of seed storage fatty acids is a necessary energy source of energy during germination of oilseed plants such as arabidopsis (Eastmond et al., 2000a), loss of this capability causes dependence on exogenous sources of energy in pxa1-l (Zolman et al., 2000). In addition, the mutant may suffer from accumulation of fatty acids and other PXA1 substrates in the cytoplasm. pxa1-l is hypersensitive to exogenous ethanol (data not shown), though it is
unknown whether ethanol normally enters the peroxisome or whether it might utilize the PXA1 channel for entry.

Despite evidence that IBA acts largely via conversion to IAA, differences exist between the physiological responses to IBA and IAA. Higher (~100 fold) concentrations of IBA are needed to elicit auxin effects (Zolman et al., 2000), and IBA is more effective than IAA for induction of lateral and adventitious roots (Hartmann et al., 1990a) and is used commercially for this purpose (de Klerk et al., 1999). Many factors could contribute to the increased efficacy of IBA for lateral root induction, including differential transport, compartmentalization, and uptake among others (de Klerk et al., 1999; Epstein and Ludwig-Müller, 1993; Ludwig-Müller, 2000). Any of these factors, or potentially specific perception of IBA, will result in an IBA-specific pattern of gene expression.

To determine the specific effects of IBA on plant gene expression, I performed pilot microarray analysis of wild-type and pxa1-1 plants mock treated and treated with IBA and IAA. Because IBA is expected to be inefficiently converted to IAA in pxa1-1, we anticipated that certain genes regulated by IBA in the mutant could represent elements of IBA response independent of IAA. In addition, comparisons between untreated pxa1-1 and wild type allowed further characterization of the in vivo effects of compromised peroxisomal fatty acid import and β-oxidation.

I purified total RNA from triplicate samples of 7-day-old, mock-treated wild type and 10-day-old pxa1-1 induced with IAA, IBA, or mock solution for 2 hours. Older pxa1-1 seedlings were examined because the mutant is slower growing that wild type; 10-day-old pxa1-1 seedlings are approximately as large as week-old wild type. RNA samples were assayed for integrity, then checked by northern blot for expression of the auxin-induced IAA1 mRNA (Figure 5.1). As expected, IAA1 was induced by both auxins in wild type (data not shown), and specifically by IAA in pxa1-1.
Figure 5.1. mRNA accumulation of the auxin-inducible transcript *IAA1*.
RNA was extracted from mock-, IAA-, and IBA-treated *pxa1-1* seedlings. A, A northern blot was performed with a portion of this RNA to reveal *IAA1* levels. B, Microarray analysis was performed with a portion of the same RNA samples as in A. Mean *IAA1* expression levels are shown relative to the mock value ± standard deviations; *n* = 3.
RNA samples were then sent to the lab of Dr. Tom McKnight for labeling and hybridization to the "whole-genome" Affymetrix ATH1 gene chip (~22,000 probe sets; Puthoff et al., 2003). Normalized quantitative results were returned for our analysis. Values for genes called "present" (based on raw abundance and statistical comparison between probe pairs for the same gene) in all conditions by Affymetrix Microarray Suite software (11,875 of 22,746 genes represented on the Affymetrix Ath1 gene chip) were included in further analysis. I converted the data into fold-change for each condition relative to mock-treated controls, calculating the relative mean and standard deviation for each gene in each condition, and excluding data points with large standard deviations (Figures 5.2, 5.3, and 5.4).

5.B. Known auxin-regulated genes

To confirm the quality of the microarray data and analysis, I examined a set of known auxin-induced genes in each condition. If the data and analysis were of good quality, IAA was expected to induce expression of certain Aux/IAA genes more strongly than IBA in pxa1-1. Indeed, expression of certain Aux/IAA genes was induced by auxins as expected (Figure 5.1 and 5.2) and is consistent with the results of the IAA1 northern blot performed using the same RNA samples (Figure 5.1). These results confirm that the analyzed microarray are consistent with previous results and suitable for the identification of genes expressed in previously-unstudied conditions.

5.C. Potential IBA-regulated genes

Having established that IBA does not detectably induce expression of IAA-induced genes in pxa1-1, I compared IBA-treated with mock-treated pxa1-1 to reveal IBA-specific alteration in gene expression. After selecting for greater than threefold changes, I identified nine arabidopsis genes regulated by IBA in pxa1-1 (Table 5.1).
Figure 5.2. mRNA accumulation of Arabidopsis genes following IAA exposure. Mock- and IAA-treated pxa1 RNA was utilized for microarray analysis. Data for genes with low expression or large standard deviations were excluded. Means from three mock and three IAA trials for 11,778 genes are shown. Diagonal bars represent threefold deviation from \( Y = X \). Selected genes with larger than threefold induction or repression are indicated. Data are expressed in arbitrary units (A.U.).
Figure 5.3. mRNA accumulation of arabidopsis genes following IBA exposure. Mock- and IBA-treated pxa/ RNA was utilized for microarray analysis. Data for genes with low expression or large standard deviations were excluded. Means from three mock and three IBA trials for 11,746 genes are shown. Diagonal bars represent threefold deviation from $Y = X$. A selected gene with larger than threefold induction is indicated. Data are expressed in arbitrary units (A.U.).
Figure 5.4. Differential mRNA accumulation in *pxa1*.
Mock-treated *pxa1* and mock-treated wild type RNA was utilized for microarray analysis. Data for genes with low expression or large standard deviations were excluded. Means from three *pxa1* and three wild type trials for 10,922 genes are shown. Diagonal bars represent threefold deviation from $Y = X$. Selected genes with larger than threefold induction or repression are indicated. Data are expressed in arbitrary units (A.U.).
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<th>description of encoded protein</th>
<th>pxA1 mock level (arbitrary units)</th>
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<th>pxA1 IAA vs. pxA1 mock</th>
<th>pxA1 mock vs. WT mock</th>
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<td>3.0 + 0.5</td>
<td>1.2 + 0.2</td>
<td>0.8 + 0.5</td>
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</table>
I then examined the relative expression of these nine genes in IAA-treated vs. mock-treated \( pxal-1 \) as well as mock-treated \( pxal-1 \) vs. mock-treated wild type (Table 5.1).

Of the genes most strongly affected by IBA exposure in \( pxal-1 \), all are upregulated and appear to encode enzymes (Table 5.1). The most highly IBA-induced genes are two putative glutathione transferases. Such transferases are often involved in molecular inactivation, and may be involved in IBA detoxification. Of these, one appears to be slightly induced by IAA, and the other does not appear to be IAA-inducible (Table 5.1). Likewise, the thioredoxin-like protein PRH26, a glycosyltransferase, and a nicotianamine synthase may be involved in responses to alteration of the chemical environment following IBA exposure. Further, a putative transporter could be involved in IBA efflux.

Thus, the microarray study has revealed several genes that may be up-regulated specifically by IBA, and may thereby contribute to IBA-specific physiological effects. It appears that, for some aspects, the IBA molecule may be itself perceived. With great strides toward understanding of IAA perception (see Chapters 1 and 7 for discussion), experiments to understand IBA perception may soon be possible.

In addition to examining gene expression following exposure to auxins, I compared wild-type and \( pxal \) mutants (Table 5.2). Three of the twelve implicated genes upregulated in \( pxal \) encode pEARLI 1-like proteins. pEARLI 1 is a protein possibly involved in stress responses (Richards and Gardner, 1995), and may indicate increased levels of toxic compounds in \( pxal \). A buildup of compounds in the cytoplasm that would normally be transported into peroxisomes via PXA1 could cause such a situation.

5.D. Future experiments with implicated genes

Key results from the pilot chip experiments could be verified and expanded by quantitative real-time PCR (Provenzano et al., 2001). These experiments would allow examination of IBA responses of many more genotypes (e.g. wild type, other
Table 5.2. Genes misregulated by more than threefold in *pxa1*.

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<tr>
<th>gene</th>
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<th><em>pxa1</em> mock vs. WT mock</th>
<th><em>pxa1</em> IAA vs. <em>pxa1</em> mock</th>
<th><em>pxa1</em> IBA vs. <em>pxa1</em> mock</th>
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<td>1300</td>
<td>3.0 + 1.2</td>
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IBA-response mutants general auxin response mutants, etc.) and other auxins and proto-auxins (2,4-D, 2,4-DB, NAA, etc.) as well as dose-response and kinetic analyses with IBA and IAA. Genes specifically upregulated by IBA might be IBA-degradation enzymes or signaling factors involved in IBA response. Gretchen Troxler and I are creating overexpression constructs for select genes upregulated specifically with IBA to test these hypotheses. If overexpression of an IBA-responsive gene confers resistance to IBA, that gene might be involved in IBA inactivation. However, if overexpression results in a constitutive auxin response (i.e. more lateral roots than wild type), the gene may be an IBA-signaling factor. The latter result would suggest that IBA is itself an auxin independent of conversion to IAA. If a gene appears to be involved in signaling rather than degradation, we will obtain an insertional knockout of that gene to characterize any auxin response phenotypes (potential knockout alleles of many of these genes have been procured).
Chapter 6: Indole-3-propionic acid, RUB modification, and the auxin response pathway

6.A. Indole-3-propionic acid causes auxin-like effects in bioassays

The IBA microarray experiments (Chapter 5) prompted a search for an indolic compound structurally related to IAA but inactive in bioassay for use as a control. An early study demonstrated that the propionic derivatives of certain synthetic auxins exert little auxin activity in several bioassays (Wain and Wightman, 1954). Because indole-3-propionic acid (IPrA) is a compound with a side chain length intermediate between IAA and IBA, previously shown to have auxin activity, the activity of IPrA was explored as a possible negative control. An experiment examining the efficacy of IPrA relative to other indolic compounds in arabidopsis bioassays was performed. Contrary to the hypothesis, IPrA proved to be a potent auxin, with an efficacy similar to IBA in bioassays (Figure 6.1). Therefore, we sought to characterize the mechanism of IPrA action.

6.A.1. axr1-12 is resistant to various auxins including IPrA

To validate that the effects of IPrA observed in bioassays resulted from auxin signaling, we tested the effects of IPrA on existing auxin response mutants. Of these, axr1-12 is a classic mutant isolated by resistance to root elongation inhibition by exogenous 2,4-D (Lincoln et al., 1990). axr1-12 is resistant to root elongation inhibition not only by IAA and IBA, but also by IPrA (Figure 6.1). In addition, axr1-12 is insensitive to hypocotyl elongation inhibition by IPrA (Figure 6.1). However, axr1-12 responds like wild type to indole-3-carboxylic acid (ICA), an indolic compound structurally similar to IAA, IBA, and IPrA (Figure 6.1). Benzoic acid is an aromatic carboxylic acid structurally related to these compounds. Likewise, axr1-12 responds like
Figure 6.1. Indole-3-propionic acid (IPrA) causes root and shoot elongation inhibition similar to other auxins.

Arabidopsis seedling root and hypocotyl lengths were measured after eight days of growth on PNS medium supplemented with the indicated indolecarboxylic acid at 22 °C under yellow light filters. ICA is indole-3-carboxylic acid; IAA is indole-3-acetic acid; IPrA is indole-3-propionic acid; IBA is indole-3-butyric acid. Points represent means +/- standard deviations; \( n \geq 9 \) except for IPrA, where \( n \geq 5 \).
wild type to the solvent in which the stocks are dissolved (Figure 6.2); normal responses to ICA and ethanol suggest a specific response to IPrA. These results demonstrate that IPrA response requires at least one component also necessary for response to other auxins.

6.A.2. *aux/iaa* mutants have altered responses to various auxins including IPrA

Several arabidopsis mutants resistant to root elongation inhibition by exogenous auxin are deficient in auxin response because of protein-stabilizing mutations in *Aux/IAA* genes (Fukaki et al., 2002; Gray et al., 2001; Hamann et al., 2002; Nagpal et al., 2000; Rogg et al., 2001; Rouse et al., 1998; Soh et al., 1999; Tatematsu et al., 2004; Tian and Reed, 1999; Wilson et al., 1990). Aux/IAA proteins are negative regulators of auxin response that are quickly degraded after auxin stimulus; however, Aux/IAA action is complicated because several factors inhibited by Aux/IAA proteins are themselves negative regulators of auxin response, resulting in a complex feedback inhibition circuit (see Chapter 1). We found that mutations in three different *Aux/IAA* genes caused altered responses to IPrA. *axr2-1* (Nagpal et al., 2000; Wilson et al., 1990), *axr3-1* (Rouse et al., 1998) and *iaa28-1* (Rogg et al., 2001) are resistant to root elongation inhibition by IPrA at high concentrations (Figure 6.3).

Auxins induce root proliferation (Chapter 1). *iaa28-1* is resistant to lateral and adventitious root proliferation by IPrA and other auxins (Figure 6.3), but still responds to IBA, suggesting a difference between IBA and IPrA induction of lateral and adventitious roots. Intriguingly, *axr2-1*, *axr3-1* and *shy2-2* are more responsive to lateral root proliferation, but less responsive to adventitious root proliferation, by IPrA and other auxins than wild type (Figure 6.3 and data not shown).
Figure 6.2. Benzoic acid and ethanol do not cause auxin-like effects.
Arabidopsis seedling root and hypocotyl lengths were measured after eight days of growth on PNS medium supplemented with either benzoic acid (an aromatic carboxylic acid) or the solvent ethanol (EtOH) at 22 °C under yellow light filters. Benzoic acid inhibits root elongation at high micromolar concentrations, but in an AXR1-independent manner inconsistent with auxin activity. Ethanol is the solvent in which auxins are dissolved in this study. Points represent means +/- standard deviations; n ≥ 9.
Figure 6.3. aux/iaa mutant responses to IPrA and IBA.
A, Arabidopsis seedling root lengths were measured after eight days of growth on PNS medium supplemented with the indicated concentration of IPrA at 22 °C under yellow light filters. Points represent means +/- standard deviations. B and C, Seedlings were grown on PNS or PNS supplemented with IBA or IPrA at 22 °C horizontally in yellow light for one day, then vertically for an additional 27 days in darkness. Number of lateral roots (roots branching from roots) and adventitious roots (roots branching from hypocotyls) were counted under a dissecting microscope. Bars represent means +/- standard deviations. n ≥ 7 except for iaa28-1 where n ≥ 3 in A, B, and C. axr3-1 was included in root proliferation experiment, however root balls too dense to count were observed.
6.A.3. IPrA-resistant mutants are also IAA-resistant

To further characterize the IPrA response, Erin Woodward and I conducted an arabidopsis mutant screen of 43,750 EMS-exposed seeds from 29 pools, 18,000 fast neutron-bombarded seeds from 6 pools, and 48,000 γ particle-irradiated seeds from 16 pools (see Chapter 2). Using a screen for reduced sensitivity to root elongation inhibition by IPrA compared to wild type, we identified 59 putative IPrA response mutants. Of 23 viable and fertile mutants isolated in this screen with reproducibly decreased auxin sensitivity, 22 were resistant to root elongation inhibition by IAA (Table 6.1), further suggesting that the two compounds utilize a single signaling pathway. The single IPrA-resistant, IAA-sensitive mutant was a fascinating find that suggested that IPrA might be chemically converted to IAA by specific plant factors or that IAA and IPrA might have at least some different modes of action. However, further characterization and cloning of the gene defective in this mutant revealed it to be involved in IAA and IPrA signaling (see ECR1 discussion below). Thus, all mutants identified in the IPrA screen are generally deficient in auxin response, or, in the case of the IPrA-specific mutant, are a special allele of a general auxin response component. This indicates that IAA and IPrA rely on a largely overlapping set of genes to elicit auxin-like effects on plant growth.

6.A.4. IPrA is bioactive across plant species

Though IPrA and IAA response rely on the same signaling machinery in arabidopsis, it remained possible that this was an arabidopsis-specific phenomenon. Indeed, previous studies suggesting low activity for propionic-side chain auxin derivatives were not performed in arabidopsis (Fawcett et al., 1960; Wain and Wightman, 1954). To test this hypothesis, I performed bioassays comparing the activity of various indolic compounds in plants other than arabidopsis. Despite numerous attempts, I was unable to achieve satisfactory results with the pea coleoptile curvature assay.
<table>
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<th>Pool&lt;sup&gt;b&lt;/sup&gt;</th>
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<sup>a</sup>EMS, ethylmethane sulfonate; FN, fast neutron.

<sup>b</sup>M2 pools preceded by L are from Lehle Seeds (Round Rock, TX).
Other bioassays were utilized to demonstrate that IPrA is an effective auxin in another dicotyledonous and a monocotyledonous plant. The dicot bean (*Phaseolus vulgaris*) was sensitive to IPrA in root elongation inhibition, similar to other auxins but not ICA (Figures 6.4 and 6.5). In addition, the monocot corn (*Zea mays*) was tested, however, these assays were not informative at the concentrations tested (Figure 6.5). Lateral root proliferation was difficult to assess in response to any auxin in both species because of large numbers on untreated bean roots and infrequent lateral roots even on auxin-treated corn roots (Figure 6.5).

6.A.5. IPrA stimulates mRNA accumulation of an IAA-inducible gene

Because IPrA exerts auxin-like effects in bioassays of more than one plant, I examined induction of a known auxin-inducible gene after IPrA treatment to determine whether IPrA activated the same molecular responses as other auxins. mRNA accumulation of *IAA1/AXR5* is induced by auxins (Abel et al., 1994). I examined *IAA1* mRNA levels following a time course of induction by various indolecarboxylic acids and found the IPrA, like IAA, induces *IAA1* mRNA accumulation (Figure 6.6). Interestingly, IBA does not induce *IAA1* accumulation over the time period studied. This result could reflect the lag time required for IBA conversion to IAA or another difference in IBA metabolism, transport, or signaling.

6.B. Some IAA- and IBA-response mutants are sensitive to IPrA

Because of abundant evidence that IPrA and IAA act by similar mechanisms, differences in response to the compounds are of critical interest. Though all IPrA response mutants are resistant to other auxins (see above), a small number of mutants resistant to other forms of auxin remain sensitive to IPrA (Figure 6.7). For example, AUX1 is a permease-type membrane protein necessary for proper IAA entry into cells (Bennett et al., 1996). Disruption of the gene in *aux1-7* causes resistance to root
Figure 6.4. IPrA is bioactive in bean root.
Surface-sterilized beans were grown on PNS supplemented with the indicated substances for seven days at 22 °C under yellow light filters. Seedlings from each condition were removed and roots were excised and photographed.
Figure 6.5. IPrA inhibits bean root elongation similar to IAA and IBA. Surface-sterilized bean and corn seeds were grown on PNS supplemented with the indicated indolecarboxylic acids for seven days at 22 °C under yellow light filters. Points represent means +/- standard deviations; $n \geq 5$ except for bean 100 nM ICA, where $n = 3$. 
Figure 6.6. IPrA induces expression of an auxin-induced transcript. Total RNA was collected from 7-day-old Col-0 seedlings grown in yellow light and induced with 100 μM of the given indolic auxin for the times shown. 7 μg RNA was subjected to gel electrophoresis, then transferred to a nitrocellulose membrane and hybridized with radiolabeled $IAA1$ DNA probe. Ethidium bromide-stained ribosomal RNA (rRNA) is shown as a loading control; all panels are from the same blot.
Figure 6.7. The auxin transport mutants doc1 and aux1 are sensitive to IPrA.
A, Arabidopsis seedling root lengths were measured after eight days of growth on PNS medium supplemented with the indicated concentrations of IPrA at 22 °C under yellow light filters. Points represent means as percentages of the mean for each genotype on unsupplemented medium +/- standard deviations; n ≥ 8. B, Arabidopsis seedlings were grown at 22 °C under yellow light filters on PNS medium for four days, then transferred to PNS (unsupplemented or with IPrA) and grown an additional four days. Bars represent means + standard deviations; n ≥ 8.
elongation inhibition by exogenous IAA and 2,4-D (Bennett et al., 1996). However, in relation to an abnormally long root on unsupplemented medium (data not shown), auxl-7 remains sensitive to IPrA application (Figure 6.7). Therefore, it is likely that IPrA uses other factors for cellular entry; alternatively, it could enter by a passive mechanism such as diffusion. As with IPrA, auxl responds normally to the synthetic auxin NAA (Yamamoto and Yamamoto, 1998), suggesting that IPrA and NAA may be similarly transported in an AUX1-independent mechanism. In addition, the auxin efflux mutant docl/tir3 has altered responses to auxins and auxin transport inhibitors (Ruegger et al., 1997). However, it is sensitive to root elongation inhibition by IPrA (Figure 6.7). These results suggest that IAA and IPrA may move across cell membranes by different mechanisms, both on entry and exit from the cell.

6.B.1 Peroxisome defects do not confer IPA resistance

Many IBA-response mutants are deficient in factors necessary for peroxisome function. Because peroxisomes are the site of fatty acid β-oxidation in plants (Gerhardt, 1992; Kindl, 1993), it appears that IBA acts after conversion to IAA by peroxisomal β-oxidation (see Chapters 3 and 4). Thus, we hypothesized that IPrA might act in an analogous way via chemical conversion to IAA by α-oxidation. Because the peroxisome is also a site of α-oxidation (Mukherji et al., 2003), we examined IPrA response in peroxisome-defective mutants. All of the peroxisomal-defective mutants tested, including pex7-1, pex5-1, pxa1-1, ibr1-1, ibr1-3, acx3-3, acx3-4, and pex6-1, remained sensitive to IPrA (Figure 6.8). These results demonstrate that peroxisomal processes, including peroxisomal α-oxidation, are not necessary for IPrA response. However, neither the peroxisome-independence of IPrA response nor the absence of mutants specifically resistant to IPrA rules out the possibility that IPrA is converted to IAA prior to signal perception. For example, IPrA could be converted to IAA by a redundantly-encoded enzyme acting outside the peroxisome.
Figure 6.8. Peroxisome defective mutants are sensitive to IPrA.
A and B, Arabidopsis seedling root lengths were measured after eight days of growth on PNS medium supplemented with the indicated indolecarboxylic acid at 22 °C under yellow light filters. Bars represent means ± standard deviations; n ≥ 11 in A, n ≥ 3 in B. C, Seedlings were grown at 22 °C under yellow light filters for four days on PNS, then transferred to PNS or PNS supplemented with IPrA and grown an additional four days. Lateral roots were counted under a dissecting microscope. Bars represents means ± standard deviations (or standard error of measurement when n = 2).
6.C. IPrA is not present at detectable levels in Arabidopsis seedlings

IPrA exerts auxin activity in bioassays across plant species. The compound acts through the previously-defined auxin response pathway based on mutant analyses and induction of gene expression. Because IPrA is structurally related to the endogenous auxins IAA and IBA, we collaborated with Dr. Jerry D. Cohen to determine whether IPrA could be detected in Arabidopsis seedlings. A recent report indicates that IPrA is present in salicylic acid (SA)-treated Arabidopsis root exudates (Bais et al., 2005); several earlier reports suggest the presence of IPrA in various plants, but these results have been questioned in light of current standards (Dr. Jerry D. Cohen, personal communication). I provided 8-day-old mock-treated and SA-treated Arabidopsis seedlings for quantitation of IAA and IPrA. IPrA was not detected in either sample, though IAA was detected in the same tissue (Barkawi and Cohen, unpublished data). The detection limit was approximately 4.74 ng/g fresh weight (Barkawi and Cohen, unpublished data). Thus, IPrA is not detectable in total tissue from 8-day-old seedlings. However, it may be produced and excreted, or produced only in certain conditions or other developmental stages.

6.D. E1 Conjugating Enzyme-Related 1

The ecr1-1 mutant (initially called W19) was isolated as the single IPrA-resistant, IAA-sensitive mutant (Table 6.1; Figure 6.9) from a screen for IPrA-resistant root elongation (see Chapter 2). The mutant is in the Col-0 accession in a lineage treated with the chemical mutagen EMS. Prior to cloning, we hypothesized that the mutant would be deficient in an enzyme required for IPrA conversion into bioactive IAA, following the precedent of prior work with IBA. However, later experiments demonstrated this hypothesis to be incorrect.
Figure 6.9. *ecr1-1* is resistant to IPrA and 2,4-D.
Arabidopsis seedling root lengths were measured after eight days of growth on PNS medium supplemented with endogenous (IAA and IBA) or synthetic (NAA and 2,4-D) auxins or IPrA at 22 °C under yellow light filters. Points represent means +/- standard deviations; *n* ≥ 8.
6.D.1. *ecrl-1* is deficient in an E1-like conjugating enzyme

The *ecrl-1* mutant was crossed to the *Ler* ecotype for mapping. Backcross analysis revealed the mutant to be recessive for reduced IPra sensitivity (Figure 6.10), facilitating mapping based on root length on IPra. *ecrl-1* was mapped to Chromosome 1 between the molecular markers nga106 and *NIT4* (Figure 6.11). New markers polymorphic between Col-0 and *Ler* ecotypes were developed within the region.

Phenotypic and candidate gene sequencing approaches were employed to eliminate several auxin-related genes in the mapping region. Because *ecrl-1* was responsive to blue light-induced shoot phototropism, the mutant was determined unlikely to be an *nph4* allele (Harper et al., 2000); data not shown). The auxin response gene *IAA28* was eliminated by sequencing the gene in *ecrl-1* (data not shown).

Finally, the candidate gene *E1 Conjugating Enzyme-Related 1 (ECRI)* was identified within the mapping region and sequenced. There was a single C to T mutation, consistent with the EMS mutagen employed, discovered in *ECRI* exon 4 (Figure 6.11). The missense mutation would cause a leucine of ECR1 to be replaced with a phenylalanine in ecr1-1. The affected residue is two residues from the catalytic site cysteine utilized for RUB conjugation to ECR1 (del Pozo et al., 1998; Figures 6.11, 6.12, and 6.13). The change of a hydrophobic leucine to a bulkier hydrophobic phenylalanine is likely to cause steric problems leading to a defect in ecr1-1 protein structure. Indeed, when Yousif Shamoo superimposed a phenylalanine on the corresponding position in the homologous human protein UBA3, the phenyl group was shown to impinge upon an adjacent α-helix (Figure 6.13).
Figure 6.10. IPrA resistance is recessive in ecr1-1.
Wild type, ecr1-1, and progeny of a plant heterozygous for ecr1-1 were grown for eight
days on PNS or PNS supplemented with 3 μM IPrA at 22 °C under yellow light filters.
Bars represent percentage of each genotype with the indicated root length; n ≥ 11 for wild
type and ecr1-1, n = 24 for ECR1/ecn1-1.
Figure 6.11. IPrA resistance is caused by a mutation in ECR1. 

ecr1-1 was crossed to the Ler accession and F2 plants resistant to IPrA were selected. IPrA-resistant plants were genotyped at polymorphic molecular markers throughout the Arabidopsis genome. A region on chromosome five was linked to the phenotype, and the ECR1 candidate gene was sequenced and found to harbor a C to T mutation, consistent with the EMS mutagen, causing a missense replacement of a leucine with a phenylalanine in the ECR1 protein. The catalytic site cysteine is indicated with an asterisk. Newly-developed markers are described in Table 6.2.
Table 6.2. New markers used in mapping *ecr1-1*.

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*Markers developed using Cereon polymorphism database.
**Figure 6.12.** ECR1 is a Uba3-like protein.

ECR1 protein sequence from arabidopsis (At) was aligned with proteins from rice (Os), Pichia pastoris (Pp), mouse (Mm), human (Hs), rat (Rn), drosophila (Dm), mosquito (Ag), neurospora (Nc), zebrafish (Dr), clostridium (Cb), roundworm (Ce), fission yeast (Sp), the fungus *Magnaporthes grisea* (Mg), and budding yeast (Sc) using the MegAlign program ClustalW algorithm (DNAStar, Madison, WI). Residues shaded black are identical in a majority of sequences; residues shaded grey are chemically similar in a majority of sequences. The location of the missense mutation in ecr1-1 is indicated with a grey asterisk; the active site cysteine for RUB attachment is indicated with a black asterisk.
Figure 6.13. The ECR1 active site.

A, Regions surrounding the active site cysteine for RUB attachment from ECR1 and related proteins were aligned using the MegAlign program ClustalW algorithm (DNASTAR, Madison, WI). Residues shaded black are identical in a majority of sequences; residues shaded grey are chemically similar in a majority of sequences. The location of the missense mutation in ecr1-1 is indicated with an arrow; the active site cysteine for RUB attachment is indicated with an asterisk. B, The image represents a structural model of the ecr1-1 defect. The phenylalanine residue present in ecr1-1 was modeled onto the equivalent position in the human ECR1 ortholog Uba3. Dots represent the van der Waals radius of isoleucine, the wild-type residue in Uba3. The fingerlike projection to the left is the active site cysteine.
6.D.2. ecr1-1 mutant phenotypes

6.D.2.A. Altered responses to IPrA, wild-type responses to IAA

The ecr1-1 mutant was isolated by reduced sensitivity to IPrA in root elongation. Analysis of M3 progeny revealed non-segregating IPrA resistance and wild-type response to IAA (Figure 6.9). ecr1-1 was the only such mutant isolated; 22 other mutants isolated by IPrA resistance were also resistant to IAA (Table 6.1).

6.D.2.B. Altered responses to other auxins

Because ecr1-1 was an anomalous mutant, we did not assume that IAA sensitivity indicated an IPrA-specific defect. Therefore, ecr1-1 was examined on various natural and synthetic auxins, and was found to have a complex auxin response defect. ecr1-1 is most compromised in IPrA response, but also is less sensitive than wild type to 2,4-D (Figure 6.9). Like with IAA, ecr1-1 responds normally to IBA and the synthetic auxin NAA (Figure 6.9). Altered responses to structurally diverse (indolic, chlorophenoxy, and napthyl) auxin compounds suggests a general deficiency of auxin response or transport. Because IAA and NAA rely on the same transport machinery to exit cells, but 2,4-D and IBA utilize distinct mechanisms for efflux (Poupard and Waddell, 2000; Utsuno et al., 1998; Zolman et al., 2000), we initially inferred that ecr1-1 might be compromised in a way that affects auxin efflux. axr1 is a mutant with a pleiotropic phenotype compromised in the heterodimerization partner for ECR1 (del Pozo et al., 2002; del Pozo et al., 1998); therefore, ecr1-1 phenotypes were compared with axr1 throughout. Interesting differences are evident between ecr1-1 and axr1-12 phenotypes and are discussed in 6.D.6. below. Likewise, rcn1 is a mutant deficient in a protein phosphatase 2A subunit that responds abnormally to many hormonal conditions (Deruère et al., 1999; Garbers et al., 1996) and is also assayed for comparison to ecr1-1.

To further examine defects in auxin responses, I tested ecr1-1 on several compounds that inhibit polar auxin transport and thereby confer auxin-like effect such as
root elongation inhibition, thought to result from local increases in auxin concentration at plant apices. Indeed, \textit{ecrl-1} has altered responses to the polar auxin transport inhibitors 1-naphthylphtalamic acid (NPA) and 2,3,5-triiodobenzoic acid (TIBA); unexpectedly, it is hypersensitive to these compounds unlike the auxin response mutant \textit{axrl-12}, which is resistant (Figure 6.14). \textit{ecrl-1} responds normally to the polar auxin transport inhibitor 9-hydroxyfluorene-9-carboxylic acid (HFCA). One mutant, \textit{pisl} (Fujita and Syono, 1997), with increased sensitivity to NPA and TIBA, but wild-type HFCA responses, has been described (Fujita and Syono, 1997), but the defective gene has not been cloned.

The mutant \textit{roots curl in NPA (rcnl)} was isolated because of abnormal root behavior when grown on NPA (Garbers et al., 1996). This mutant is defective in a protein phosphatase 2A subunit (Deruère et al., 1999) and has increased auxin transport (Rashotte et al., 2001). \textit{rcnl} hypersensitivity to NPA has been suggested by biochemical results, but has not previously been demonstrated in bioassays. Using our standard assay conditions, I found that the \textit{rcnl} mutant is hypersensitive to NPA and HFCA (Figure 6.14).

6.D.2.C. Altered responses to other phytohormones and environmental stimuli

The \textit{ecrl-1} mutant is deficient in response to several auxins, therefore it might be expected to have altered responses to other stimuli. A broad survey of hormone and environmental responses reveals a complex phenotype with many impaired responses. In addition to altered auxin responses, \textit{ecrl-1} is hypersensitive to the phytohormones cytokinin (benzyladenine) and brassinosteroid (epibrassinolide; Figure 6.15). The mutant has characteristics associated with increased ethylene biosynthesis or response, such as short, thick hypocotyls in darkness (Figure 6.16). However, \textit{ecrl-1} has wild-type responses to ethylene-induced root elongation inhibition (Figure 6.15). The mutant is less sensitive than wild type to methyl jasmonate (MeJA; Figure 6.15). \textit{ecrl-1} is
Figure 6.14. *ecrl-1* is hypersensitive to a subset of auxin transport inhibitors. Seedling root lengths were measured after eight days of growth on PNS medium supplemented with the indicated auxin transport inhibitors at 22 °C under yellow light filters. Points represent means +/- standard deviations; *n* ≥ 10.
Figure 6.15. Responses to various phytohormones are abnormal in ecr1-1.
Seedling root lengths were measured after eight days of growth on PNS medium supplemented with the indicated plant growth regulators at 22 °C under yellow light filters. Points represent means +/- standard deviations; n ≥ 7.
Figure 6.16. *ecrl-1* light responses.

A, Seedlings were grown on PNS medium at 22 °C under yellow light filters for one day, then an additional four days in darkness. Hypocotyls were then measured. Bars represent means ± standard deviations; *n* ≥ 11. B, Seedlings were grown at 22 °C in white light for one day, then an additional 4 days in darkness; *n* ≥ 11. C and D, Seedlings were grown on PNS medium plates wrapped in the indicated colors of cellophane at 22 °C under white light for eight days; *n* ≥ 10.
hypersensitive to gibberellin (gibberellic acid (GA₃) and cytokinin (benzyladenine; Figure 6.15)

In addition to hormonal responses, ecr1-1 was tested in various environmental conditions. The mutant is more responsive than wild type to root elongation inhibition in red light (Figure 6.16). ecr1-1 hypocotyls are shorter than wild type when grown at 28 °C (Figure 6.17), a temperature that promotes auxin accumulation. In addition, the mutant makes fewer lateral roots than wild type when grown at 22 or 28 °C or when grown on the synthetic auxin NAA (Figure 6.18). However, ecr1-1 responds to induction of lateral root proliferation by elevated temperature and exogenous auxin (Figure 6.18). Despite these developmental defects, ecr1-1 is normal in many ways: ecr1-1 hypocotyls are normal in the light, root elongation responds normally to many substances, and adult plants are indistinguishable from wild type (data not shown).

6.D.3. Double mutant phenotypes

Because many signaling pathways are compromised in ecr1-1, we crossed it with other mutants to tease apart the specific contribution of ECR1 to each response of interest. Analysis of double ecr1-1 double mutants is ongoing. Because of the presumed partial defect in ecr1-1, it will be interesting to learn whether double mutants with axr1 alleles have additive phenotypes. Such additive phenotypes would indicate that both ecr1-1 and the axr1 allele are partial loss-of-function or that the El subunits have functions independent of heterodimerization with each other.

6.D.4. ECR1 is required for seedling development

After identifying ECR1 as the defective gene, I obtained two independent T-DNA alleles from the Salk collection (Alonso et al., 2003). Both ecr1-2 and ecr1-3 heterozygous lines give rise to seedlings that germinate but then arrest development as small, red-colored seedlings (data not shown). This phenotype is consistent with the
Figure 6.17. Responses to growth at elevated temperature.
Seedling hypocotyl lengths were measured after eight days of growth on PNS medium under yellow light filters at 22 °C or 28 °C. Bars represent means ± standard deviations; \( n \geq 10 \).
Figure 6.18. *ecr1-1* responses to lateral root induction by NAA and elevated temperature.
Seedlings were grown for eight days on PNS or PNS supplemented with NAA under yellow light filters at 22 °C (or 28 °C where indicated). Bars represent means + standard deviations; \( n \geq 9 \).
seedling lethal phenotypes of some other mutants defective in the RUB modification pathway (Wei and Deng, 1992). Though two independent lines have the seedling-lethal phenotype and I have been unable to isolate homozygous lines of either mutation, linkage analysis needs to be performed to demonstrate that the developmentally arrested plants are homozygous for ecr1 disruption. These data suggest that the viable ecr1-1 is indeed a special allele, as expected from the rarity with which it was recovered following mutagenesis.

6.D.5. ECR1 is a RUB-activating enzyme

ECR1 is the heterodimer binding partner of AXR1 (del Pozo et al., 2002; del Pozo et al., 1998), a protein long implicated in auxin response (Lincoln et al., 1990). Together, the two proteins constitute an E1-type activating enzyme for the small ubiquitin-like protein Related to Ubiquitin 1 (RUB; del Pozo et al., 2002). ECR1 and AXR1 are homologous to the N-terminal and C-terminal regions, respectively, of ubiquitin E1 proteins. The ECR1 subunit contains domains necessary for RUB activation by conjugation to AMP as well as the cysteine to which RUB is conjugated (Figure 6.19; del Pozo et al., 1998). Once conjugated to the ECR1-AXR1 heterodimer, RUB is transferred to the RUB1 Conjugating Enzyme (RCE1), which acts like a ubiquitin E2 protein (Dharmasiri et al., 2003b), and is then transferred to the Skp1-Cullin-F-box (SCF) ubiquitin E3 complex Cullin subunit (del Pozo and Estelle, 1999). Cullin modification by RUB plays a regulatory role, affecting many SCF-mediated processes, and influences Cullin protein stability (del Pozo et al., 2002). In an antagonistic role, the COP9 signalosome catalyzes RUB removal from Cullin proteins (Schwechheimer and Deng, 2001).

Because ecr1-1 harbors a missense mutation, it is unclear whether the phenotypes result from a loss or gain of ECR1 function. Therefore, I examined RUB modification of CUL1 in wild type, ecr1-1, two loss-of-function alleles of axr1 including a strong
Figure 6.19. Cullin accumulation is abnormal in ecrl-1.
Total protein from 2-day-old arabidopsis seedlings was extracted, subjected to SDS-PAGE, and transferred to a membrane. The membrane was hybridized with α-CUL1 antibody and visualized by chemoluminescence (Chapter 2). CUL1 protein is ~90 kDa. The higher molecular weight RUB-modified form of CUL1 is indicated.
(axr1-12) and weak (axr1-3) loss-of-function allele, and cop9, a mutant deficient in RUB
removal from CUL1 (Lyapina et al., 2001; Schwechheimer et al., 2001). Western blots
of seedling protein with CUL1 antibody revealed increased unmodified CUL1
accumulation in ecr1-1 and axr1 alleles; conversely, I found less unmodified CUL1 in
cop9 (Figure 6.19). Therefore, ecr1-1 is a loss-of-function allele that affects CUL1
protein in the same way as axr1.

Interestingly, CUL1-RUB levels appeared unchanged in all genotypes assayed. It
is an unexpected finding that loss of RUB activating enzyme activity affects CUL1 levels
rather than CUL1-RUB; a previous report has been interpreted to show a decrease in the
proportion of RUB-modified versus free CUL1 in axr1-12, though some western blots
presented could be interpreted differently (del Pozo et al., 2002). Though there is a
paucity of data to explain this phenomenon, it is possible that RUB modification
negatively regulates CUL1 stability, while a mechanism may exist to maintain a constant
pool size of CUL1-RUB.

6.D.6. ECR1 and AXR1 are involved in many plant developmental processes

Defects in AXR1 lead to diverse phenotypes including reduced sensitivity to
auxin-induced root elongation inhibition (Table 1.4). Likewise, ecr1-1 causes many
altered hormonal responses (see above); however, numerous discrepancies exist between
the phenotypes of axr1 and ecr1-1 mutants. Because the proteins function as a
heterodimer and CUL1 is similarly affected in the mutants, this phenotypic disagreement
is surprising. Further, the phenotypes are opposite in some assays. For example, axr1-12
is resistant to root elongation inhibition by auxin transport inhibitors NPA and TIBA,
whereas ecr1-1 is hypersensitive to these compounds (Figure 6.14). In addition, axr1-12
hypocotyls are long in darkness, although ecr1-1 hypocotyls are short relative to wild
type (Figure 6.16). In phenotypes shared by axr1-12 and ecr1-1, relative severity also is
not consistent. For example, axr1-12 is less sensitive than ecr1-1 to the synthetic auxin
2,4-D, but *ecr1-l* is less sensitive than *axr1-l2* to IPrA (Figure 6.9). In addition, *axr1-l2* is a viable knockout mutant (the mutation converts a glutamine to a stop codon, truncating 23% of the protein), whereas *ecr1* knockout alleles are seedling lethal (see above).

The discrepancies between *axr1* and *ecr1-l* mutant phenotypes could be explained in several ways. The arabidopsis genome contains an additional gene with high similarity to *AXR1*, but only a single apparent *ECR1*. Thus, it is possible that the two AXR1 proteins regulate plant development in different ways. However, no function is known for ECR1-AXR1 other than RUB activation and transfer to RCE1. It remains possible that other pathways exist, and that different ECR1-AXR combinations feed differentially into the alternative RUB modification pathways. Another possible explanation invokes the many different and often opposing signaling pathways affected by SCF complexes. The phenotypes ultimately observed could be exquisitely sensitive to levels of RUB activation efficiency and thereby difficult to conceptualize.

**6.E. Aux/IAA proteins may be targets of RUB modification**

Members of the Auxin/Indole-3-acetic acid (Aux/IAA) protein family are involved in negative regulation of auxin response (Tiwari et al., 2003). Following auxin stimulus, destruction of these negative regulators is mediated by ubiquitination via SCF<sup>TIR1</sup> (Gray et al., 2001; Zenser et al., 2001). TIR1 is the specificity-determining F-box component of SCF<sup>TIR1</sup>; auxin stimulates interaction between TIR1 and Aux/IAA proteins bringing Aux/IAA proteins to the SCF for ubiquitination and degradation (Gray et al., 2001); see Chapter 1 for detailed discussion.

**6.E.1. Aux/IAA proteins share sequence identity with CUL proteins**

Cullin proteins are targets of RUB modification (del Pozo and Estelle, 1999), which in turn regulates SCF activity (del Pozo et al., 2002). p53 is the only protein other
than members of the Cullin family known to be a target for RUB modification (Xirodimas et al., 2004). It is difficult to predict which lysine or lysines will be utilized for ubiquitin attachment in a target protein, perhaps because thousands of E3 proteins are responsible for ubiquitin target selection. Unlike ubiquitin targets, Cullin proteins share a highly conserved domain in which a single lysine is modified by RUB (del Pozo and Estelle, 1999). However, it is difficult to know whether this conservation is part of a RUB modification site or serves some other conserved Cullin function. Intriguingly, lysines necessary for mammalian p53 RUB modification fall within a domain conserved with the RUB modification domain of Cullin proteins (Figure 6.20), suggesting that additional RUB modification sites in plants might be predicted computationally.

To identify other arabidopsis proteins that might be modified by RUB, I performed a translated nucleotide BLAST search (Altschul et al., 1990) of the residues surrounding the RUB attachment site lysine in arabidopsis CUL1 against the entire arabidopsis genome. One of the few proteins returned by the query was an Aux/IAA protein. Alignment of all Aux/IAA proteins with cullins revealed several residues are conserved between many Aux/IAA protein family members and CUL1 in this region, including the CUL1 lysine modified by RUB (Figure 6.20). Further, the domain is spatially conserved, located near the C-terminus of both protein types. However, some Aux/IAA proteins terminate prior to this region. Of the Aux/IAA proteins identified in mutant screens, 8 of 9 bear this region including the internal lysine residue. A portion of a bipartite nuclear localization sequence is found within the region of interest in Aux/IAA proteins (Abel and Theologis, 1995). Because Aux/IAA proteins associate with an SCF complex that bears a RUB E3 enzyme (Gray et al., 2002), this would be a potential mechanism for either ubiquitin or RUB modification of Aux/IAA substrates. Aux/IAA proteins in association with SCF$^{SK1}$ are proximal to a destruction mechanism; resultant instability could account for the failure to previously identify Aux/IAA-RUB molecules.
Figure 6.20. An Aux/IAA domain similar to the Cullin RUB conjugation site. Fragments of Cullin and Aux/IAA protein sequences were aligned using the MegAlign program ClustalW algorithm (DNAStar, Madison, WI). Residues shaded black are identical in a majority of sequences; residues shaded grey are chemically similar in a majority of sequences. The location of the RUB-modified Cullin lysine is indicated with an asterisk.
For this reason, even ubiquitinated Aux/IAA proteins, though long assumed, have not been demonstrated.


To determine whether the Aux/IAA lysine identified by sequence homology to Cullin proteins is important for Aux/IAA function, I performed site-directed mutagenesis to disrupt it. A construct containing the Aux/IAA gene *IAA28* under control of the endogenous promoter fused with a myc epitope was modified to encode an arginine in place of the conserved lysine (See Chapter 2). Thus, the basic chemistry of the residue is preserved, but the ability to form a covalent bond with RUB is abolished. This construct will be transformed into wild-type and *iaa28-I* mutants to test for altered auxin response phenotypes that would indicate the importance of the conserved lysine.

The Aux/IAA domain containing this lysine has been previously implicated in dimerization (Kim et al., 1997; Ulmasov et al., 1997b). Because Aux/IAA proteins heterodimerize with and inhibit Auxin Response Factor proteins (ARFs; Rogg and Bartel, 2001), RUB modification in this region could facilitate ARF protein release from Aux/IAA heterodimers, freeing ARF proteins from potential inadvertent destruction by SCF^{TIR1}. Therefore, one hypothesis is that rendering an Aux/IAA protein RUB resistant would lead to auxin-responsive ARF degradation and thereby cause auxin resistance. It will be interesting to learn whether this lysine is important for Aux/IAA function. If phenotypes are observed, further biochemical studies will be necessary to establish whether RUB modification is the essential function of the conserved lysine.
Chapter 7: Conclusions and future prospects*

7.A. Peroxisome biogenesis and auxin

Proper peroxisome function is critical for animal and plant health. Plant peroxisomes house enzymes for fatty acid catabolism and the activation of molecules to produce phytohormones. Plants are particularly good organisms for the study of peroxisomal dysfunction because they utilize many of the same biogenesis factors as humans and, to the best of our knowledge, feel no pain.

7.A.1. Peroxisomal targeting signal receptors

A fascinating corollary between mammalian and plant peroxisome biogenesis is interaction between receptors for the two targeting signals that mark proteins for import into the peroxisomal matrix. In mammals, a specific isoform of the PTS1 receptor PEX5 interacts with the PTS2 receptor PEX7 (Braverman et al., 1998). Plants produce a PEX5 isoform containing the region needed for interaction with PEX7 in mammals (Chapter 3). Indeed, mutation of the putative PEX7-binding region results in specific PTS2 protein import defects. It remains to be determined whether plants also make a ‘short’ form of PEX5 lacking the PEX7 interaction region. RT-PCR experiments of plant tissues at different developmental stages could reveal the presence of an alternatively-spliced mRNA. Even in the characterized mammalian system, the functional implications of possible developmental differences have not been explored.

Using a reverse-genetic approach, I demonstrated that arabidopsis PEX7 is necessary for PTS2 protein import into peroxisomes and that plant PEX7 function is relevant in vivo. The pex7-1 mutant is resistant to the proto-auxin IBA, which is converted in peroxisomes into the active auxin IAA (Zolman et al., 2000). This phenotype was rescued by 35S promoter-driven overexpression of PEX7. The pex7-1

* Portions of this chapter have been previously published (Woodward and Bartel, 2005b).
mutant is not notably impaired when grown without light and sucrose, suggesting that the endogenous fatty acid β-oxidation needed for seedling development in these conditions is minimally affected. The relatively weak pex7-1 phenotype may be attributable to the nature of the lesion; the T-DNA insertion in pex7-1, though within the 5′ UTR of a cDNA (Schmid et al., 2003), allows some expression of presumably fully-functional PEX7. When we examined PTS2-GFP localization in pex7-1, we found little punctate fluorescence, indicating a severe block in PTS2 protein import. However, the apparent severity of pex7-1 in this assay could result from flooding the cells with 35S-expressed PTS2-GFP. Indeed, endogenous PTS2-targeted thiolase import is only slightly affected in pex7-1, revealing the sensitivity of IBA resistance and PTS2-GFP localization assays in detecting peroxisome defects.

Because PEX7 appears to deliver cargo to the peroxisome via interaction with PEX5 in arabidopsis (Johnson and Olsen, 2003; Nito et al., 2002; Sparkes and Baker, 2002) as it does in mammals (Braverman et al., 1998; Dodt et al., 2001; Matsumura et al., 2000), we also examined PTS1- and PTS2-protein import in the existing pex5-1 allele (Zolman et al., 2000). Interestingly, PTS1-GFP localization was properly punctate in pex5-1, whereas PTS2-GFP was diffuse. Moreover, PTS2-containing thiolase was inefficiently imported into pex5-1 peroxisomes. There is one other existing pex5 mutant of this type, a CHO cell line with a PTS2-specific defect resulting from the inability of PEX7 to bind mutant pex5 (Matsumura et al., 2000). Remarkably, the lesion in the CHO mutant pex5 changes an analogous serine to that mutated in pex5-1. Moreover, both are mutated to hydrophobic amino acids, leucine in arabidopsis pex5-1 and phenylalanine in the CHO mutant. The characterization of these two pex5 alleles with PTS2-specific defects demonstrates the critical importance of this conserved serine for PEX7 binding and suggests an ancient origin for PEX7-PEX5 interaction. Further, whereas the CHO cell line was isolated in a screen that selected for PTS2-deficient import (Matsumura et al., 2000), arabidopsis pex5-1 was isolated from a screen for IBA resistance, which can
yield a variety of peroxisomal defects (Zolman and Bartel, 2004; Zolman et al., 2001a; Zolman et al., 2001b; Zolman et al., 2000); this result may suggest that PTS2 proteins are particularly important for IBA metabolism in seedlings. Indeed, the peroxisome-defective mutant ped2/pex14 is deficient in IBA (Monroe-Augustus, 2004) and 2,4-DB (Hayashi et al., 2000) response as well as PTS2-protein import (Hayashi et al., 2000). Though the IBA-response mutant pex6 is defective in PTS1 import (Zolman and Bartel, 2004), it remains unknown whether this mutant is also deficient in PTS2-protein import.

Though an N-terminal arabidopsis PEX5 fragment lacking the region containing the pex5-l mutation has been shown to interact with PEX7 in a yeast two-hybrid assay, the interaction was less robust than with full-length PEX5 (Nito et al., 2002). Further, the interaction was enhanced in a PEX5 fragment containing the region mutated in pex5-l (Nito et al., 2002). Thus, the region we implicate in PTS2 import is necessary, but may not be sufficient, for the PEX5-PEX7 interaction driving PTS2 import in vivo.

Whereas both pex7-l and pex5-l single mutants are peroxisomally deficient in only the most sensitive assays, combining the two defects yields a severely-affected double mutant with numerous developmental abnormalities, likely resulting from reduced PEX7 expression combined with inefficient PEX7-PEX5 interaction (Figure 7.1). PTS2-targeted thiolase import is severely affected in pex7-l pex5-l, while PEX7 expression and PEX5 levels are similar to those in the respective single mutants; this result is consistent with the double mutant harboring two partial loss-of-function mutations in the same pathway. pex7-l pex5-l requires exogenous sucrose for seedling establishment, but not for growth as an adult plant. The sucrose dependence of this mutant demonstrates the necessity of PTS2 protein import for utilization of seed-storage lipids by seedlings before photosynthesis is established, a process of specialized peroxisomes termed glyoxysomes (Beever, 2002).
Figure 7.1. Cooperative import of PTS1 and PTS2 proteins in Arabidopsis.
A, PEX7 binds both PTS2 protein cargo and the PTS1 protein receptor PEX5. Delivery of PTS2 protein into the peroxisome is dependent on complex formation with PEX5. PTS2 protein cargo is necessary for peroxisomal processes of fatty acid β-oxidation and conversion of IBA into the active auxin IAA. B, Functional PEX7 may be expressed at reduced levels in the pex7-1 mutant, resulting in reduced PTS2 protein import, IBA resistance, and decreased lateral root number. C, PEX7 binding with pex5-1 is inefficient, resulting in decreased PTS2 protein import and IBA resistance. D, Two partial defects are combined in pex7-1 pex5-1 to nearly eliminate PTS2 protein import, resulting in severe developmental defects.
Though the PTS2 signal sequence is less common than PTS1, it is more common in plants than other organisms (Johnson and Olsen, 2001; Mullen, 2002), and is greatly over-represented among β-oxidation enzymes highly expressed during the glyoxysome-dependent seedling establishment phase (Kamada et al., 2003). PTS2-bearing β-oxidation enzymes with peak expression in seedlings (Kamada et al., 2003) include the acyl-CoA synthases LACS6 and LACS7 (Fulda et al., 2002), the acyl-CoA oxidases ACX2 (Hooks et al., 1999) and ACX3 (Froman et al., 2000), and the thiolase PED1/KAT2 (Germain et al., 2001; Hayashi et al., 1998). Among these enzymes, ped1/kat2 mutants (Germain et al., 2001; Hayashi et al., 1998) and the lacs6 lacs7 double mutant require exogenous sucrose for seedling establishment (Fulda et al., 2004). Although both LACS6 and LACS7 have functional PTS2 signal sequences, LACS7 also possesses a functional PTS1 sequence, and the lacs6 single mutant is sucrose-independent (Fulda et al., 2002), making these proteins unlikely causes of sucrose dependence in the PTS2-import deficient pex7-1 pex5-1. However, sucrose dependence in pex7-1 pex5-1 could result from mislocalization of the PTS2-targeted PED1/KAT2 to the cytosol. In contrast to these β-oxidation enzymes, all known enzymes directly involved in photorespiration in green leaf peroxisomes bear PTS1 signal sequences (Reumann, 2002); the PTS2-independence of photorespiration could account for the healthy green coloration of the pex7-1 pex5-1 mutant, which is unlike the pale-colored pex14 and pex6 mutants (Hayashi et al., 2000; Zolman and Bartel, 2004).

*pex7-1 pex5-1* plants germinated on sucrose and transferred to soil grow into stunted adults that produce few normal seeds, only a minority of which germinate, even when supplied with sucrose. Thus, PTS2 import is required not only for seedling establishment, but also for vegetative growth and normal seed development. Strikingly, the ssel mutant, which is defective in PEX16, a peroxin implicated in early steps in peroxisome formation, was isolated because it bears ~90% inviable shrunked seeds (Lin et al., 1999). Further, null mutations in PEX2 and PEX10 are lethal at stages prior to seed
maturation (Hu et al., 2002; Schumann et al., 2003; Sparkes et al., 2003). Thus, the high frequency of shrunken and inviable seeds in the pex7-1 pex5-1 double mutant again implicates peroxisomes in proper seed development and reveals the necessity of PTS2 protein import for this process.

Those pex7-1 pex5-1 seeds that do germinate often display fused cotyledons. Several arabidopsis mutants with defective cotyledon separation have been described, and these link cotyledon development with auxin response. The pin-formed1 mutant has frequent cotyledon fusion and is defective in an auxin efflux facilitator that is necessary to establish auxin gradients in developing embryos (Gälweiler et al., 1998). The monopteros mutant likewise has variably fused cotyledons and is defective in an auxin response factor that interprets auxin gradients (Hardtke and Berleth, 1998). The cup-shaped cotyledon double mutant cuc1 cuc2 was isolated on the basis of cotyledon fusion, and is defective in functionally-redundant transcription factor genes (Aida et al., 1997; Takada et al., 2001) that are misregulated in monopteros and pin-formed1 (Aida et al., 2002). Thus, auxin signaling is critical for proper cotyledon development.

The requirement of auxin response for proper embryonic symmetry, the requirement for peroxisome function to allow IBA conversion into active IAA, and the reduced auxin response phenotype of pex7-1 pex5-1 implicate IBA as a critical auxin reservoir during embryogenesis. Though IBA levels have not been quantified in seeds, IBA is present in seedling tissue at nearly the levels of free IAA (Ludwig-Müller et al., 1993).

PEX7 is necessary for peroxisome function in mammals and yeast (Mukai et al., 2002; Rehling et al., 1996), and characterization of the first plant pex7 mutant reveals a reduction in PTS2 protein import into peroxisomes. We also show that the sole described plant pex5 mutant is defective in PTS2-, but not PTS1-protein import. pex7-1 pex5-1 double mutants have severe PTS2 import defects, several developmental abnormalities, and a high frequency of embryonic death and deformities, some of which may result from
defective IBA metabolism. It will be interesting to observe the phenotypes of hypothetical \textit{pex5} mutants specifically defective in PTS1 rather than PTS2 import, which will reveal the roles for PTS1 import in plant development.

Thus, plants and mammals appear to share a system for peroxisomal matrix protein import in which two peroxisomal targeting sequences are present and both rely on PEX5 for import into the organelle. It is important to note, however, that the PTS2 pathway is absent from the model animals \textit{Drosophila melanogaster} and \textit{Caenorhabditis elegans} (Chapter 3; Motley et al., 2000). Likewise, the model eukaryote, the fungus \textit{Saccharomyces cerevisiae}, utilizes PEX7 and PTS2 proteins in a PEX5-independent parallel pathway unlike animals and plants.

\textbf{7.A.2. Other \textit{arabidopsis} peroxisome biogenesis and IBA-response mutants}

Highlighting the critical importance of peroxisomes in plant development, loss of some peroxisome biogenesis factors causes embryonic death in \textit{arabidopsis} mutants (Chapter 1). For example, insertional disruption of the RING finger peroxins PEX2 and PEX10 in \textit{arabidopsis} do not survive early development (Hu et al., 2002; Schumann et al., 2003; Sparkes et al., 2003). Homozygous \textit{pex10} seeds form embryos that arrest at early stages of development (Schumann et al., 2003; Sparkes et al., 2003). However, it has been shown that \textit{pex2} heterozygous plants produce shorter seed pods than wild-type, suggesting that \textit{pex2} homozygous embryos do not survive long enough to reserve a space in the structure (Hu et al., 2002). Contrary to this, I found that insertional disruption of \textit{pex2} does not affect seed pod length, but instead causes development of abnormal seeds (Chapter 4). I obtained almost identical results indicating embryonic arrest with the previously-uncharacterized RING finger peroxin mutant \textit{pex12}.

Further experiments could be performed to characterize embryonic development defects in \textit{pex2} and \textit{pex12}. For example, microscopic imaging of embryos will establish the developmental stage at which homozygous mutant embryos arrest. In addition,
abnormalities in the mutant embryos may suggest a role for these peroxins in normal plant development. To test the correlation between PEX2 and PEX12 gene dysfunction and embryonic death, arrested embryos will be dissected from the seeds pods for genotypic correlation with the mutant allele. Likewise, wild-type genes will be transformed into heterozygous plants to directly test the hypothesis that the lack of PEX2 or PEX12 function causes embryonic death.

Additional biochemical experiments could be employed to characterize the roles of PEX2 and PEX12 in peroxisome biogenesis and plant development. First, it will be important to demonstrate that, as with PEX2 (Hu et al., 2002) and other peroxins, PEX12 is localized on the peroxisomal membrane. In addition, the RING finger domain present in PEX2, PEX10, and PEX12 suggests a role in conjugation of ubiquitin or a related protein to other proteins (Freemont, 2000). Biochemical studies of protein-protein interactions and potential self-modification may reveal a role for these proteins in ubiquitination or a related process, as well as identify any target proteins. Recent studies have demonstrated that the PTS1 receptor Pex5p is a target for ubiquitination in yeast, and that the Pex5p modification plays a vital role in delivery of Pex5p cargo to the peroxisome (Kiel et al., 2005; Kragt et al., 2005; Platta et al., 2004). Further, loss of Pex2p, Pex10p, or Pex12p reduces yeast Pex5p ubiquitination, implicating these peroxins in the process in yeast (Kragt et al., 2005). It will be interesting to learn whether plant PEX5 is also ubiquitinated, and whether any or all of the RING finger peroxins play a role in the process in plants.

Isolation and characterization of arabidopsis mutants defective in other hypothetical peroxisome biogenesis factors is ongoing (Chapter 4). The TILLING strategy for isolation of point mutations within a selected gene (McCallum et al., 2000) is a particularly promising avenue for these reverse-genetic projects in which complete loss-of-function alleles of many players are dead. Such mutants have been isolated (Chapters 3 and 4), but careful phenotypic analysis awaits several generations of backcrossing to
reduce unlinked mutations. In addition, forward genetic approaches to understanding peroxisome function in plants have not been exhausted (Chapter 4). These unbiased genetic screens hold promise for identification of new, and potentially plant-specific, peroxisome biogenesis factors as well as peroxisomal matrix protein workhorses. IBA has proven a valuable tool in plant peroxisome analysis (Monroe-Augustus, 2004; Zolman, 2002; Chapters 2 and 3), and will continue to be employed in assays of plant peroxisome function.

7.B. Compounds with auxin-like activity

Several natural and synthetic molecules influence plant development in ways that earn classification as an auxin (Chapter 1). Among these, IBA is a commercially-utilized compound (Hartmann et al., 1990b) once thought to be a synthetic auxin, and only recently discovered in vivo (Bartel et al., 2001; Epstein and Ludwig-Müller, 1993; Ludwig-Müller, 2000). IBA response depends upon peroxisomal function, at least in part, suggesting that IBA acts through conversion to IAA in a peroxisomal β-oxidation-like process (Zolman et al., 2000; Figure 7.2). Indeed, peroxisomal function in general, and PTS2 protein import in particular, may be essential for endogenous IBA metabolism required during embryonic development (Chapter 3).

Despite evidence that IBA acts via conversion to IAA, a possibility for IAA-independent IBA responses remains (Figure 7.2). Several IBA-response mutants have reduced IBA sensitivity, but are not completely insensitive (Zolman and Bartel, 2004; Zolman et al., 2001b; Zolman et al., 2000). Therefore, I performed microarray analysis of the pxa1 mutant, and pxa1 after stimulation with IAA or IBA (Chapter 5). Several apparent enzymes are induced by IBA in the mutant background. The proteins could catalyze IBA inactivation or another process influencing or influenced by IBA levels. In addition, I have identified the genes most misregulated in the pxa1 mutant when compared to wild type, which may ultimately reveal some in vivo effects of blocked
**Figure 7.2. Plant responses to indolecarboxylic auxins.**

IAA is a potent auxin with well-characterized influences on gene expression. IBA is a less-potent natural auxin that acts at least in part through conversion to IAA (Zolman et al., 2000). IBA is generated from IAA \textit{in vivo} (Ludwig-Müller and Epstein, 1992). IPrA is an auxin active at comparable concentrations to IBA in Arabidopsis (Chapter 6). Dashed arrows indicated hypothetical conversions or signaling cascades.
β-oxidation. Identification of these genes allows experiments to examine the roles they play in plant development.

In a quest to find an indolecarboxylic acid molecule structurally similar to IAA and IBA but lacking auxin bioactivity, I discovered IPrA to be a surprisingly effective auxin (Chapter 6). IPrA is active in the same concentration range as IBA, and both are less effective than IAA. IPrA bioactivity is not limited to Arabidopsis; I observed root elongation inhibition in other plants as well. Testing these auxins in an optimized assay for auxin activity in a monocotyledonous plant will reveal the evolutionary conservation of IPrA bioactivity.

IPrA bioactivity may be achieved by stimulating gene expression patterns in the same manner as IAA. Both IAA and IPrA stimulate mRNA accumulation of the Aux/IAA gene IAA1. Interestingly, IBA does not induce IAA1 message accumulation, at least on the same time scale, suggesting a difference in IBA response kinetics or the molecular mechanisms underlying IBA versus IAA and IPrA response.

To further characterize IPrA responses in Arabidopsis, Erin Woodward and I performed a large-scale genetic screen of mutagenized plants to identify individuals compromised in IPrA responses. All IPrA-response mutants identified, with only one exception, were also resistant to exogenous IAA, suggesting substantial overlap between the mechanisms of IAA and IPrA responses.

In a surprising twist, analysis of the single IPrA-resistant, IAA-sensitive mutant led to ECR1, a protein at the heart of auxin signaling. ecr1-1 is compromised in the enzyme E1-CONJUGATING ENZYME-RELATED 1, a heterodimer partner with AUXIN-RESISTANT 1 (AXR1) that activates the small ubiquitin-related molecule RUB (del Pozo et al., 2002; del Pozo et al., 1998; Leyser et al., 1993). I found that accumulation of the RUB target protein CUL1 is altered in ecr1-1. However, introduction of wild-type ECR1 into ecr1-1 will be necessary to directly test whether all of the phenotypes observed are caused by the mutation of the ECR1 gene.
ecrl-1 has a complex auxin phenotype, resistant to IPrA and 2,4-D but sensitive to IAA, IBA, and NAA. Further, ecrl-1 is hypersensitive to some auxin transport inhibitors. These results suggest that RUB regulation of the auxin signaling apparatus (Figure 7.3) does not influence responses to all auxins equivalently. RUB modification impacts a protein involved in many plant signaling cascades, so it is not surprising that ecrl-1 has phenotypes on other phytohormones as well as auxin. Examination of double mutants deficient in RUB modification in combination with genes specifically involved in response to a particular hormone may reveal the relative contribution of RUB modification to each implicated pathway.

Thus, the number of molecules in the auxin class continues to grow. IBA appears to influence expression of some genes independently of conversion to IAA. Confirmation of IBA regulation of these genes and characterization of individual genes will reveal their roles in IBA response and plant development. For example, if some genes are involved in IBA inactivation or conjugation, overexpression of the genes may reduce IBA sensitivity. Further, bioinformatic comparison of the upstream regions of IBA-regulated genes may allow identification of an IBA-response element, and, ultimately, the proteins involved in IBA recognition and response. Similar microarray experiments may help understand differential responses to IPrA, namely differential efficacy of IAA and IPrA and the specificity of ecrl-1 defects. Examination of mutants specifically defective in response to one or a subset of auxins may reveal other players with differential functions.

7.C. Auxin biology

Auxin is a critical phytohormone. Complex and redundant regulation of IAA abundance, transport, and response interact in an intricate system of auxin utilization that achieves a variety of purposes in plant development. As a result, the study of auxin
Figure 7.3. Auxin signaling and RUB cycling.

A, An activating ARF protein binds an AuxRE promoter element via an N-terminal DNA binding domain (DBD). Under low-auxin conditions, an Aux/IAA repressor binds the activating ARF via heterodimerization between Aux/IAA and ARF domains III and IV. B, Auxin promotes Aux/IAA domain II-TIR1 association, bringing the Aux/IAA protein to the SCF$^{TIR1}$ complex for ubiquitination (Ub) and subsequent destruction by the 26S proteasome. The activating ARF, with a Gln-rich (Q) middle domain, is then freed to promote auxin-induced gene expression. The ubiquitin-related protein RUB is activated by ECR1/AXR1, transferred to RCE1, and conjugated to CUL1 to regulate SCF activity. RUB is cleaved from CUL1 by a subunit of the COP9 signalosome, a protein complex that may function as a proteasome lid. See text of Chapter 1 for references.
biology is impacting our understanding of an astounding variety of processes, from regulated protein degradation to signal transduction cascades, from organelle biogenesis to plant morphogenesis. Despite prodigious historical and ongoing auxin research, many of the most fundamental original questions remain incompletely answered.

Redundancy is a key theme in auxin metabolism. IAA is produced both from the amino acid tryptophan and from an independent pathway utilizing a Trp precursor (Normanly et al., 1993). IAA can be stored in certain conjugated forms, which can be hydrolyzed (Bartel and Fink, 1995) or as IBA, which can be β-oxidized (Zolman et al., 2000) to regenerate free IAA. Further, the isolation of a protein bearing covalently attached IAA (Walz et al., 2002) suggests an exciting new area for auxin storage research. A variety of auxin degradation products have been isolated from plants (Östln et al., 1998), suggesting redundant auxin inactivation pathways. Future work will establish the relative contributions to auxin homeostasis from each of these redundant mechanisms.

In addition, tantalizing clues are emerging about the subcellular compartmentalization of auxin metabolism and response. Differential compartmentalization and controlled entry and exit from organelles may provide regulatory points for auxin and its precursors, although it is unknown where free IAA and its storage forms accumulate within the cell. Trp biosynthetic enzymes are plastidic (Radwanski and Last, 1995), whereas several potential downstream Trp-dependent IAA biosynthetic enzymes, including YUCCA and AAO1, are apparently cytoplasmic, and the subcellular location of Trp-independent IAA biosynthesis remains a mystery. Interestingly, two enzymes that catalyze a step in IAA biosynthesis, CYP79B2 and CYP79B3, have chloroplast targeting signals (Hull et al., 2000); perhaps compartmentalization aids channeling common intermediates to either IAA or indolic glucosinolates. Directly comparing IAA and Trp precursor and metabolite levels in

*yucca, sur1*, and *sur2* mutants may reveal the importance of this potential
compartmentalization. The subcellular location of IAA-conjugate biosynthesis and IAA degradation are also unknown. IAA-conjugate hydrolase sequence are consistent with ER retention (Bartel and Fink, 1995; Davies et al., 1999). Also, β-oxidation of IBA to yield IAA apparently takes place in peroxisomes (Zolman et al., 2000). Finally, it is unclear how many different sites in the cell can perceive auxin, though at least one auxin signaling apparatus is apparently entirely soluble (Dharmasiri et al., 2003a). Examination of mutants compromised in functions of individual organelles (Chapters 3, 4, and 5) will aid in elucidation of the contributions of each compartment to auxin metabolism and response.

The effectors through which auxin signaling influences growth and development are currently being identified. These will include the direct targets of ARFs and downstream effectors of these genes, combined with any non-transcriptional auxin responses. Transcript changes associated with lateral root production in response to auxin are beginning to be identified (Himanen et al., 2004); some of these are likely to be ARF targets. For example, lateral root proliferation in response to auxin is mediated by the transcription factor encoded by the auxin-induced NAC1 gene, which acts downstream of TIR1 (Xie et al., 2000).

In addition to auxin effectors downstream of TIR1, some processes appear to be mediated by auxin independent of the TIR1 signaling pathway. For example, auxin leads to rapid acidification of the extracellular space by enhancing plasma membrane ATPase activity, allowing cell wall loosening and thereby enabling cell growth (Hager, 2003). This process may be regulated by auxin, at least in part, via a signal transduction pathway acting in parallel to the SCF^{TIR1} pathway (Hager, 2003).

Auxin is also proving useful in the study of various cellular processes of general interest. For example, IBA and 2,4-DB response mutants are revealing key steps in peroxisome biogenesis and function, and the search for genes influencing IAA-amino acid conjugate responses has revealed new players in metal homeostasis (Lasswell et al.,
200; Magidin et al., 2003). Further, studies of disrupted auxin transport reveal that the actin cytoskeleton, unidentified endosomal compartments, and a rapid vesicular trafficking are all involved (Surpin and Raikhel, 2004). Finally, the study of auxin-response mutants has provided multiple new insights into the roles of SCF complexes in ubiquitin-mediated proteolysis.

A tremendous area of future research will be the characterization and understanding of interactions between different phytohormones and environmental stimuli. The channeling of several hormone signaling pathways through specific SCF complexes with shared components is one possible arena for cross-talk. In addition, many mutants with altered response to auxin and other stimuli remain to be fully characterized or understood.

The recent identification of microRNAs and their targets in plants (Bartel and Bartel, 2003) has revealed intriguing links to auxin signaling. Several of these tiny riboregulators, which direct negative regulation of complementary mRNAs (Bartel, 2004), target messages implicated in auxin responsiveness. For example, miR167 targets ARF6 and ARF8 mRNAs (Kasschau et al., 2003; Rhoades et al., 2002), which encode activating ARFs (Ulmasov et al., 1999a), and miR160 targets ARF10, ARF16, and ARF17 mRNAs (Kasschau et al., 2003; Rhoades et al., 2002), which have not been functionally characterized but resemble repressing ARFs. Moreover, miR393 targets mRNAs encoding TIR1 and the three most closely-related F-box proteins (Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004). In addition to microRNAs influencing auxin signaling, microRNAs are likely to aid in signal interpretation. For example, miR164 targets CUC1 and CUC2 (Laufs et al., 2004; Mallory et al., 2004), which establish organ boundaries in embryos and flowers (Aida et al., 1997) and are negatively regulated by PIN1 and PINOID (Furutani et al., 2004). Thus microRNAs, like ubiquitin-mediated protein degradation, are likely to be post-transcriptional regulators defining the extent of auxin signaling and response during development and in response to environmental cues.
The single most pressing question of auxin biology remains the identity of the auxin receptors. Tremendous progress has been made in this area, starting with the identification of SCF$^{\text{TIR1}}$ as a critical conduit of auxin response (Gray et al., 1999; Leyser et al., 1993). Recent results suggest that auxin causes modification of the TIR1 F-box, or a tightly associated protein, to increase TIR1 interaction with Aux/IAA repressors, bringing them to the SCF complex to be targeted for destruction (Kepinski and Leyser, 2004). However, the nature of the modification, as well as the proximity to the auxin receptor, remain undetermined. While it is likely that SCF$^{\text{TIR1}}$ mediates many transcriptional responses to auxin, it remains to be seen whether there are SCF$^{\text{TIR1}}$-independent transcriptional auxin responses, or whether this signaling complex also mediates rapid responses to auxin that are not transcriptional.

Most importantly, identification of the auxin receptor will allow rigorous examination of the properties necessary for a molecule to act directly as an auxin. When a functional auxin signaling pathway including each receptor can be reproduced in an alien system (i.e. animal cells or cell extract), different compounds can be added to test the ability of each to activate each receptor or labeled compounds can be tested for binding to the purified receptor directly. It is possible that if a family of auxin receptors exists, a subset (or each individual protein) will have specific ligand affinity profiles. Such a model could help explain why some mutants, such as ecrl-1, have reduced sensitivity to only some auxin-like molecules. Regardless of the results, identification of the auxin receptor coupled with examination of mutants with altered responses to specific auxins will be the keys to identification of the true molecular nature of the critical plant hormone auxin.
Appendix

A. *axes3-1*, an arabidopsis mutant hypersensitive to growth at elevated temperature

A.1. Introduction

Because plants are sessile, they must stand and face environmental challenges that would cause an animal to move and seek safety. One such challenge is heat: most plants rely on solar energy for sustenance, but life in the sunshine can be quite hot. Therefore, plants rely on orchestrated responses — some general to other organisms, some plant-specific — to heat stress that enable survival.

High heat causes many challenges to a plant, including protein denaturation, increased water loss, and the many downstream effects of these primary problems. However, the first step in protection against heat damage is to sense the problem. Though little is known about thermosensory mechanisms, temporary exposure to intermediate temperatures can confer tolerance to a normally lethal exposure to a higher temperature (Hong and Vierling, 2000). Thus, plants sense and adjust to temperature increases.

At least one way that plants achieve heat tolerance is through the action of heat stress transcription factors (HSFs). HSF proteins bind to elements in the promoter regions of heat-induced genes and promote transcription following heat stress (Zhang et al., 2003). Many target genes are molecular chaperones involved in the maintenance of proper protein folding, a class of proteins important for heat tolerance in many organisms.

However, plants also express a class of heat shock proteins present only in plants, lower eukaryotes, and bacteria (Lee et al., 2005). These proteins, members of the
ATPases associated with various cellular activities (AAA ATPase) class, mediate refolding of proteins that are difficult substrates for classical heat shock proteins (Goloubinoff et al., 1999). A screen for mutants hypersensitive to heat stress following acclimation has implicated one such AAA ATPase, AtHSP101, as critical for thermotolerance in arabidopsis (Hong and Vierling, 2000).

In addition to these responses, phytohormones play roles in heat tolerance. For example, the hormone abscisic acid is necessary for closure of stomatal pores (Artsaenko et al., 1995), a process critical to minimize heat-induced water loss. In addition, elevated temperature induces accumulation of endogenous auxin (Gray et al., 1998). This auxin, in turn, leads to exaggerated hypocotyl elongation and lateral root proliferation (Gray et al., 1998; Rogg et al., 2001). The mechanism and relevance of auxin accumulation in response to heat remain unresolved.

A.2. Materials and methods

See Chapter 2 for general plant growth conditions and assays. Procedures specific to the *axs3*-1 mutant are described below.

A.2.A. *axs3*-1 mutant isolation

*axs3*-1 was identified in an EMS-mutagenized seed line. Originally, the seed line carrying *axs3*-1 (originally called X255) was of interest because of a putative mutation causing increased lateral root proliferation and altered response to auxin transport inhibitors (Magidin, 2002). I observed hypersensitivity to growth at elevated temperature segregating in the background of this putative mutant. Homozygous lines hypersensitive to hypocotyl elongation at 28 °C were isolated and backcrossed to wild-type Col-0.
A.2.B. Phenotypic analyses

Sensitivity to heat was determined in three ways: first, seeds were surface sterilized, stratified overnight, and grown on PNS in Percival incubators under yellow light filters at 22 °C or 28 °C for 8 days, then root and hypocotyl lengths were measured.

Second, plants were sterilized and stratified as above, exposed to white light in a 22 °C incubator for 2 hours, then covered in aluminum foil and grown in darkness in a 30 °C incubator. After 4 days, plants were removed and hypocotyls were measured.

A final assay for sensitivity to transient heat shock was performed by preparing seeds as above, growing them for five hours under white light at 22 °C to promote germination, then covering the plates in aluminum foil. Plants were grown in darkness for 3 days, and then transferred to a 37 °C oven for 30 to 180 minutes before being returned to the 22 °C incubator. Other plants were incubated at 37 °C for 90 minutes to acclimate them to the heat, then moved to 45 °C for an additional 30-120 minutes, and returned to the 22 °C incubator. All plants were recovered for 2 days in the 22 °C incubator, and then hypocotyls were measured.

Mannitol, sorbitol, mannose, and geldanamycin PNS plates were prepared from filter-sterilized water stocks (except DMSO for geldanamycin) as with other substances in Chapter 2.

A.3. axs3 phenotypes

A.3.A. High temperature hypersensitivity

axs3-1 was isolated from an EMS-mutagenized Col-0 accession background thought to carry a mutation conferring auxin hypersensitivity. Auxin accumulates in seedlings grown at 28 °C to a higher level than seedlings grown at 22 °C and causes exaggerated hypocotyl elongation at 28 °C (Gray et al., 1998; Rogg et al., 2001). Therefore, seeds from this line were plated and grown at 28 °C under yellow light. Some
individuals thought to be auxin hypersensitive arrested when grown at 28 °C (Figure A.1). High temperature hypersensitivity was not linked to auxin hypersensitivity in these lines; further, the background was later determined not to carry an auxin hypersensitive trait (data not shown). Thus, axs3-1 represented a previously uncharacterized mutation.

Besides continuous growth at elevated temperature, further assays were conducted to characterize the nature of axs3-1 heat hypersensitivity. Because initial assays were performed in the light, it was unclear whether light conditions were necessary for the hypersensitive response. Therefore, axs3-1 was grown in darkness at 30 °C. In these conditions, axs3-1 development arrested after minimal growth, while wild-type seedlings developed apparently unhindered (Figure A.2).

Because axs3-1 develops similar to wild type when grown at 22 °C, it was not clear whether the mutant was hypersensitive only to prolonged heat exposure, or would suffer from transient heat stress as well. Plants are able to tolerate transient heat stress when first acclimated in a moderately elevated temperature (Hong and Vierling, 2000). If the axs3-1 phenotype resulted from a temperature-sensitive allele of an essential housekeeping gene, I hypothesized that the mutant would suffer more from prolonged heat stress than wild type, but that the mutant might be able to quickly recover from transient heat stress, potentially able to refold a misfolded protein or express more of the affected protein. Therefore, plants were acclimated at 37 °C for 1.5 hours, then transferred to 45 °C, a temperature lethal to wild-type plants. After various lengths of exposure, plants were removed and recovered at 22 °C for 2 days. In this assay, both axs3-1 and wild type were increasingly affected by increasing periods of exposure to 45 °C conditions. However, axs3-1 responses parallel wild-type in the assay (Figure A.3), revealing that axs3-1 is not hypersensitive to transient heat shock following acclimation. Therefore, it is likely that axs3-1 harbors a temperature sensitive allele of a required gene rather than a defect in generalized high temperature stress response.
Figure A.1. *axis3* is hypersensitive to elevated temperatures.
Wild-type and *axis3* seedlings were grown on PN or PNS medium under yellow light filters at 22 °C except where indicated for eight days. Bars represent mean root lengths + standard deviations; *n* ≥ 9.
**Figure A.2.** *axs3* is hypersensitive to elevated temperatures in darkness.

Wild-type and *axs3* seedlings were grown on PN medium under white light at 22 °C for two days to promote germination, then grown for five additional days in darkness at 30 °C. Bars represent mean hypocotyl lengths ± standard deviations; *n* = 14.
Figure A.3. *axs3* responds like wild type to transient heat shock.
Wild-type and *axs3* seedlings were grown under white light at 22 °C for five hours, then in darkness for three days. At this time, plants were transferred to 37 °C for the indicated time, then returned to 22 °C or transferred to 45 °C for the indicated time. Plants were allowed to recover for an additional two days at 22 °C. Points represent mean root lengths +/- standard deviations; *n* ≥ 11.
A.3.B. Hormone-related responses

Because *axs3-I* is hypersensitive to high temperatures, high temperatures are linked to increased auxin accumulation, and *axs3-I* was thought to be derived from an auxin-hypersensitive parent, I sought to determine whether *axs3-I* was itself hypersensitive to auxin. *axs3-I* was not hypersensitive to root elongation inhibition by auxin (Figure A.4). Likewise, the secondary root proliferation was induced by auxins in *axs3-I* to a similar level as wild type (data not shown). Further, if *axs3-I* high temperature hypersensitivity was caused by excess auxin production or heightened auxin response, it would follow that high temperature might cause both root elongation inhibition and lateral root proliferation in the mutant. However, *axs3-I* makes remarkably fewer lateral roots than wild type when grown at 28 °C, suggesting that the problem is a developmental arrest rather than an auxin-mediated dysfunction.

Despite the absence of a link between *axs3-I* phenotypes and auxin, the defect could potentially result from misregulation of another hormone. To test this, the mutant was grown in the presence of cytokinin (benzyladenine), methyl jasmonate, salicylic acid, and gibberellic acid (GA$_3$). *axs3* root elongation at 22 °C was enhanced by gibberellin (Figure A.4). Therefore, I examined gibberellin-induced elongation at both moderate and elevated temperature. *axs3* responds normally to GA, increasing hypocotyl elongation at both high and low concentrations; however, GA is not sufficient to restore wild-type development at elevated temperature (Figure A.5). Therefore, *axs3* phenotypes are unlikely to be caused by deficient gibberellin response. Mutant roots were shorter on cytokinin than wild type (Figure A.6), but whether this reflects hypersensitivity is unclear because *axs3* has shorter roots than wild type on unsupplemented growth medium. Mutant responses to methyl jasmonate and salicylic acid appear similar to wild type (Figure A.6).
Figure A.4. *axis3* responses to various conditions.
Wild-type and *axis3* seedlings were grown under yellow light filters at 22 °C unless indicated for eight days. Plants were grown on supplemented or unsupplemented PNS unless indicated. eBR is epibrassinolide; GA3 is a gibberellic acid. Bars represent mean root lengths versus root length on unsupplemented medium at 22 °C + standard deviations; $n \geq 9$. 

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Figure A.5. *axs3* hypocotyls respond to gibberelic acid.
Wild-type and *axs3* seedlings were grown on unsupplemented or supplemented PNS under yellow light filters at 22 °C unless indicated for eight days. Bars represent mean hypocotyl lengths versus root length on unsupplemented medium at 22 °C + standard deviations; *n* ≥ 9.
Figure A.6. *axis3* phenotypes in various conditions.
Wild-type and *axis3* seedlings were grown on unsupplemented or supplemented PNS under white light at 22 °C unless indicated for eight days. BA is the cytokinin benzyladenine; MeJA is methyljasmonic acid; SA is salicylic acid. BA, SA, LiCl, NaNO₃, mannitol, and sorbitol plates were prepared without sucrose. PN to 10 μM SA represents plants grown for four days on unsupplemented PN, then transferred to PN containing 10 μM SA for an additional four days of growth. 0.1% PN is normal growth medium diluted tenfold but solidified with the usual amount of agar; this solution was supplemented to 0.5% [w/v] sucrose (the usual sucrose concentration in PNS) to make 0.1% PNS. Bars represent mean root lengths ± standard deviations; n ≥ 9.
A.3.C. Osmotic stress responses

Exhaustive examination of *axs3-1* phenotypes revealed an alteration in sensitivity to high osmotic potential (Figure A.6). *axs3-1* plants grown on high mannitol or sorbitol concentrations are unaffected at levels that inhibit development in wild type (Figure A.7). The dose-response curve does not reveal obvious resistance in *axs3-1*, however, because the mutant root remains shorter than wild type at all concentrations: it remains possible that osmotic stress affects wild-type growth via the pathway compromised in *axs3-1*. Decreased sensitivity to osmotic stress has previously been observed in generally stress-tolerant (Xiong et al., 2004) and hormone response (Ruggiero et al., 2004) mutants. However, *axs3-1* is sensitive to exogenous salts (Figure A.4). In addition, no hormone response phenotypes have been observed, though abscisic acid, previously correlated with osmotic stress (Ruggiero et al., 2004), has not been tested. Understanding this complex osmotic phenotype will require further phenotypic and molecular characterization.

Altered responses to sucrose were also observed in *axs3* (Figures A.1, A.4, and A.6). Repeatedly, the mutant appears healthier on exogenous sucrose than without sucrose. Therefore, I performed a concentration curve to examine responses to various levels of exogenous sucrose. Though *axs3* root elongation inhibition was less severe than wild type, it followed the same trend (Figure A.8). In contrast, the sucrose-dependent mutant *pex6* (Zolman and Bartel, 2004) benefitted from sucrose concentrations inhibitory to both wild type and *axs3* (Figure A.8). Therefore, it is possible that altered *axs3* sucrose response is related to osmotic stress phenotypes rather than metabolic dysfunction.
Figure A.7. Osmotic stress response in *axs3*.
Wild-type and *axs3* seedlings were grown on unsupplemented or supplemented PNS under white light at 22 °C for eight days. Points represent mean root lengths +/- standard deviations; n ≥ 11.
Figure A.8. Sucrose response in *axs3*.
Wild-type and *axs3* seedlings were grown on PN containing the indicated concentrations of sucrose under white light at 22 °C for eight days. The peroxisome biogenesis mutant *pex6* is shown for comparison. Note that 50 mM sucrose is beneficial to *pex6*, but inhibitory to both *axs3* and wild type. Points represent mean root lengths +/- standard deviations; *n* ≥ 10.
A.3.D. Geldanamycin

The compound geldanamycin inhibits certain heat shock proteins that function in conformational stabilization of other proteins (Queitsch et al., 2002; Rutherford and Lindquist, 1998). Exposure to geldanamycin has been shown to reveal “hidden” variation, that is, differences in amino acid sequences of proteins that are not phenotypically visible under normal growth conditions (Queitsch et al., 2002; Rutherford and Lindquist, 1998). Thus, if axs3-I were compromised in this aspect of stress tolerance, the mutant response to geldanamycin might differ from that of wild type. However, axs3-I root elongation is inhibited by geldanamycin similarly to wild type (Figure A.9).

A.3.E. Other conditions

To further characterize axs3-I phenotypes and gain perspective on the possible defect, I tested responses to other stress conditions (Figure A.4 and A.6). However, axs3-I responded like wild type in these conditions, again suggesting that axs3-I is not generally deficient in stress response. In addition, phototropism and gravitropism assays revealed normal tropic responses (data not shown).

A.4. Mapping

axs3-I was outcrossed to Ler andWs accessions. I utilized polymorphic molecular markers to map the mutation to a region of Arabidopsis chromosome 1 between the markers F5M6 and T9L6 developed in this study (Figure A.10). This region includes one obvious candidate gene, the heat stress transcription factor HSF8. However, I was unable to identify mutations in this gene by direct sequencing of HSF8 from axs3-I. Heat induces expression of the cell wall modification gene XTR4 (Iliev et al., 2002). Therefore, XTR4, CslD6, and a glucanase gene were sequenced from axs3-I; however, no mutations were found. The molecular identity of AXS3 remains unknown.
Figure A.9. *axis3* responds normally to geldanamycin. Wild-type and *axis3* seedlings were grown on PNS containing the indicated concentrations of geldanamycin at 22 °C for eight days. Points represent mean root lengths ± standard deviations; *n* ≥ 11.
Figure A.10. *axs3* mapping.
Recombination mapping was employed to localize *axs3* to a region on arabidopsis chromosome 1. Molecular markers are indicated and fractions represent the number of recombinant chromosomes over the total examined. New markers are described in Table A.1. Numbers indicate the position relative to the tip of chromosome 1 in nucleotides. Asterisks indicate the locations of candidate genes.
Table A.1. New markers used in *axs* mapping.

<table>
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<th>Chr</th>
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<td>At1g30710</td>
<td></td>
<td>103 70</td>
<td>4</td>
</tr>
<tr>
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*Markers developed using Cereon polymorphism database.
†Marker developed using TAMU database.
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