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CHARACTERIZATION OF Arabidopsis thaliana MUTANTS DEFECTIVE IN AUXIN METABOLISM

by

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

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ABSTRACT

Auxins are an important class of phytohormones that play a critical role in various aspects of plant growth and development such as cell elongation, cell division, apical dominance, tropisms, and fruit ripening. The most abundant naturally occurring auxin is indole-3-acetic acid (IAA). IAA is found in plants both as the free acid and in conjugated forms. It is believed that the balance between IAA biosynthesis, metabolism, and transport determines the actual levels of IAA in a cell at any given time, but the molecular mechanisms controlling this regulation are largely unknown. In my thesis project, an IAA-alanine resistant mutant from Arabidopsis thaliana, iar4, has been phenotypically characterized and mapped to chromosome 1. Three mutants potentially defective in the last step of IAA biosynthesis have also been characterized. One of these mutants has been tested for complementation with a gene encoding a putative aldehyde oxidase, AtAO4.
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List of Abbreviations

AO, aldehyde oxidase
BAC, bacterial artificial chromosome
CAPS, cleaved amplified polymorphic sequences
cM, centimorgan
EMS, ethyl methanesulfonate
IAA, indole-3-acetic acid
IAA-Ala, IAA alanine
IAA-Leu, IAA leucine
IAA-Phe, IAA phenylalanine
IAALD, indole-3-acetaldehyde
IALD, indole-3-aldehyde
IAM, indole-3-acetamide
IAN, indole-3-acetonitrile
IPA, indole-3-pyruvic acid
SSLP, simple sequence length polymorphisms
kb, kilobasepair
Trp, tryptophan
μM, micro molar
mM, milimolar
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INTRODUCTION

Auxins Are an Important Class of Plant Hormones

The study of auxin began with Darwin, who found that a substance in the tip (named auxin later) was essential to the uneven growth of coleoptiles in response to light. Since then, biochemical and physiological investigations have provided us with valuable knowledge of the physiological functions of auxin. Most of the knowledge comes from the study of indole-3-acetic acid (IAA), the predominant form of auxin (Davies, 1995) (Figure 1), which is involved in various aspects of plant development and growth such as cell elongation, cell division, and tropisms (Thimann, 1977).

Auxin is believed to affect hypocotyl elongation and formation of an apical hook at the top of the hypocotyl by controlling cell elongation. Auxin-overproducing Arabidopsis seedlings containing the iaam transgene, which encodes a tryptophan (Trp) monooxygenase converting Trp to indole-3-acetamide (IAM), an IAA precursor, showed dramatically longer hypocotyls in the light than wild type plants, and observation of cross-sections of these hypocotyls proved that the increased hypocotyl growth is due to cell elongation (Romano et al., 1995). When grown in the light at high temperature (29°C), Arabidopsis seedlings also have longer hypocotyls, and this elongation has been found to be mediated by auxin since these seedlings contain elevated IAA levels and IAA response and transport mutants or IAA-underproducing iaal (IAA-lysine synthetase) transgenic seedlings do not have longer hypocotyls at high temperature (Gray et al., 1998). It has been suggested that the differential distribution of auxin on the two sides of the hypocotyl causes differential elongation of the cells on each side, therefore forming the apical hook. In the Arabidopsis hookless1 (hls1) mutant, apical hook formation was abolished and expression patterns of two auxin-regulated genes were altered (Lehman et al., 1996). The HLS1 gene has been cloned and found to encode a putative N-acetyltransferase. The function of the HLS1 protein is unknown, but there is speculation that HLS1 could regulate the activity or stability of a protein involved in auxin transport (Lehman et al., 1996). It has also been
Figure 1: Structures of indole-3-acetic acid (IAA); IAA-myo-inositol, an ester-linked conjugate of IAA; IAA-alanine, an amide-linked conjugate of IAA.
observed that high concentrations of IAA inhibit hypocotyl elongation in the dark (Bartel and Lasswell, unpublished data). All the above data suggest that auxin mediates cell elongation and apical hook formation in hypocotyls.

Plant gravitropism, the growth of roots towards gravity and shoots away from gravity, is also believed to involve the action of auxin. By studying auxin transport across root caps, Young et al. (1990) found that auxin movement in roots is asymmetric (preferentially downward). Laser ablation of cap cells abolished the gravitropic response of the roots, indicating that differential distribution of auxin in the roots may be essential to the response (Blancaflor et al., 1998). Arabidopsis eir1 mutant roots failed to respond to gravity when grown on agar plates oriented vertically, but had wild-type IAA sensitivity (Roman et al., 1995). The cloning and characterization of EIR1 suggest that it encodes a protein required for the efflux of auxin from the cells of the root tip into the elongation zone (Luschnig et al., 1998).

Auxin also regulates cell division, therefore it affects the outgrowth of lateral buds and lateral roots. By uncoupling auxin and ethylene effects in transgenic tobacco and Arabidopsis plants, Romano et al. (1993) showed that in iaaM transgenic plants, apical dominance is increased and numbers of lateral buds are reduced. In the Arabidopsis auxin-overproducing rooty (rty) mutant, lateral root growth is dramatically increased (King et al., 1995). By studying an allele of rty, superroot1(surl), Boerjan et al. (1995) showed that lateral root growth was initiated from pericycle cell divisions. Consistent with this, decreased response to auxin leads to reduced lateral root formation. In Arabidopsis auxin-resistant mutants axr1, aux1, and axr4, fewer lateral roots are formed than in wild type, and in double mutants consisting of two of these three single mutants there are further reductions in lateral root formation (Hobbie and Estelle, 1995; Timpte et al., 1995).

Despite the wealth of information on the physiological functions of auxin, the regulatory mechanisms of auxin metabolism are hardly known. During the course of evolution, plants have developed various ways to produce auxin. It is the cooperation and
coordination of these pathways that determine the actual levels of auxins in a cell at any developmental stage to optimize plant growth (Normanly, 1997). The two pathways that were investigated in this project are conjugation/deconjugation and de novo synthesis.

These studies have utilized Arabidopsis thaliana, a very useful and popular model system for investigating a broad range of topics in plant biology. This small, diploid, self-fertilizing plant has a small genome with a low frequency of repetitive DNA, which facilitates genetic manipulation and gene cloning (Meyerowitz, 1992).

Hydrolysis of IAA Conjugates Is an Important Aspect of IAA Metabolism

In plants, about 95% of the IAA is conjugated via ester linkages to sugars or myo-inositol, or via amide linkages to amino acids, peptides, or proteins (Bandurski et al., 1995) (Figure 1). IAA conjugates have been proposed to have several functions.

First, IAA conjugates provide storage for free IAA. Using isotope labeling techniques, it has been found that free IAA is rapidly decarboxylated in pea stem segments while decarboxylation of IAA conjugates is slow, and application of IAA-alanine (IAA-Ala) has the same effects as IAA on plant growth (Hangarter and Good, 1981). Application of different IAA conjugates to bean stem sections revealed that the biological activity of IAA conjugates is correlated with the ability of plant tissue to hydrolyze these conjugates to produce free IAA (Bialek et al., 1983). By studying the turnover of indolic compounds in maize, Epstein et al (1980) found that it is not de novo synthesis but conjugate hydrolysis that supplies the developing seedlings with IAA. Therefore, IAA conjugates can serve as reservoirs of inactive IAA.

Second, IAA conjugation is suggested as a means of regulating free IAA levels. The introduction of the bacterial iaaL gene into Nicotiana tabacum (tobacco) and potato leads to morphological abnormalities such as leaf epinasty, reduced apical dominance, and decreased rooting late in development (Romano et al., 1991; Spena et al., 1991). These altered morphologies are correlated with reduced free IAA and increased IAA-lysine levels.
Moreover, the abnormal phenotypes in *iaal* tobacco transgenic plants can be reversed by *iaam*-mediated IAA overexpression, thus confirming that it is indeed the perturbed auxin homeostasis that causes the changes on morphology (Romano et al., 1991). Such evidence suggests that IAA conjugates are very critical to the levels of free IAA.

It has also been suggested that IAA conjugates function in IAA transport. Epstein et al. (1980) have shown that in maize seedlings, IAA inositol enters the shoots approximately 400 times faster than free IAA.

While IAA conjugates play an important role in IAA regulation, the enzymes and genes involved in conjugation and deconjugation are largely unknown. The first gene involved in conjugation, *iaglu*, was cloned from maize. *iaglu* encodes an enzyme showing both IAA-glucose-forming and -hydrolyzing activity (Szerszen et al., 1994). Enzymes hydrolyzing IAA-glucose have also been identified in maize and potato (Kowalczyk et al., 1990; Jakubowska et al., 1993). Partial purification of an IAA-amide hydrolase recognizing IAA-Ala, IAA-phenylalanine (IAA-Phe), and IAA-Trp has been carried out in carrot cells (Kuleck and Cohen, 1993). However, most of hydrolyzing enzymes are unstable and difficult to purify. Therefore, genetic approaches have become a useful tool to study IAA conjugates.

Since *Arabidopsis thaliana* is such a powerful model system for genetic studies of plants, a great deal of work on IAA metabolism has been done in it. Mutant screens for IAA conjugate-resistant plants, which showed long roots in the presence of high concentration of IAA conjugates, were performed. Campanella et al. (1996) isolated two IAA conjugate resistant mutants, *icr1* and *icr2*, which were resistant to IAA-Phe, IAA-Ala, and IAA-glycine. The *ILR1* (for IAA-Leucine-resistant) gene, which encodes an enzyme that hydrolyzes IAA-Leu and IAA-Phe *in vitro* was isolated by positional cloning of an IAA-Leu resistance locus (Bartel and Fink, 1995). Other IAA conjugate hydrolase genes, *ILL1* (for IAA-Leu-resistant like), *ILL2* (Bartel and Fink, 1995), *ILL3* and *ILL5* (Davies et al., 1999) were identified by homology to the *ILR1* gene. All ILL proteins except ILL5 have
been purified to investigate their substrate specificities (Davies and Bartel, unpublished data).

Three Arabidopsis IAA-Ala resistant mutants, *iar1*, *iar2*, and *iar4* were also isolated. *IAR3* has been cloned and found to encode an enzyme hydrolyzing IAA-Ala (Davies et al., 1999). *IAR1* has been cloned and it encodes a protein showing features of a transmembrane protein that may be involved in IAA conjugate transport (Lasswell and Bartel, unpublished data). The isolation of multiple complementation groups that confer IAA-Ala resistance suggests that the conversion of IAA-Ala to IAA is regulated by several gene products. In this project, *iar4* was characterized and genetically mapped as a first step towards positional cloning of the *IAR4* gene.

**IAA Biosynthesis**

In addition to hydrolysis of IAA conjugates, *de novo* synthesis of IAA is a way to produce IAA for plant growth and development. However, the study of IAA biosynthesis has been complicated by the following problems: 1) low levels of IAA in intact cells; 2) non-enzymatic degradation of indolic compounds to yield IAA; 3) bacterial contamination; and 4) disrupted compartmentalization of cells during *in vitro* assays (Kawaguchi and Syono, 1996).

Despite these difficulties, the improvement of techniques for stable isotope dilution analysis, coupled with gas chromatography-mass spectrometry (GC-MS), provides a very useful tool for quantitation of IAA from small amounts of tissue. In bean seedlings, incubation of isolated axes on labeled Trp lead to high incorporation of Trp into IAA (Bialek et al., 1992), while in *Lemna gibba*, only low incorporation of labeled Trp into IAA was detected (Baldi et al., 1991). By studying the regulation of IAA biosynthetic pathway in carrot cell cultures, Michalczuk et al. (1992) showed that incubation with 2,4-dichlorophenoxyacetic acid, a synthetic auxin, leads to high incorporation of Trp into IAA while normal embryogenesis uses a non-tryptophan pathway to produce IAA. All the above
data support the idea that IAA biosynthesis can be both Trp-dependent and Trp-independent.

Mutants in IAA biosynthesis are also available. A maize Trp auxotrophic mutant, *orange pericarp*, produces 50 times more IAA than wild type, and addition of Trp does not affect the amounts of IAA produced in the mutant or normal seedlings (Wright et al., 1991). Two Arabidopsis Trp biosynthesis mutants, *trp2* and *trp3*, also accumulate high levels of IAA (Normanly et al., 1993). These data strongly support the existence of Trp-independent IAA synthesis. Interestingly, *trp2* and *trp3* mutants also contain high levels of indole-3-acetonitrile (IAN), a possible IAA precursor (Normanly et al., 1993). The cloning of genes encoding nitrilases capable of converting IAN to IAA (Bartel et al., 1994) support this possibility.

Mutants of nitrilase have been isolated from Arabidopsis. The *nlt1* mutant showed three- to fourfold increased root growth compared with that of wild-type in the presence of 30 μM IAN (Normanly et al., 1997). However, steady state levels of free IAA, total IAA, and IAN were similar in both *nlt1* and wild-type plants. This is not surprising because nitrilase has several isozymes in Arabidopsis (Bartel and Fink, 1994) so that lack of activity of one enzyme may be compensated by the activity of other enzymes.

Several microbes in the rhizosphere (the area around the roots of plants) also produce IAA, which is implicated in the stimulation of growth or pathogenesis of plants (Loper et al., 1986). Therefore, intensive research has been done in microorganisms on IAA biosynthesis and it has been suggested that IAA biosynthetic pathways may be similar in microbes and plants (Patten et al., 1996). This work has provided very useful information about the intermediates in Trp-dependent IAA biosynthetic pathways. Several Trp-dependent routes have been proposed, and they are named for their intermediates, such as the IAM pathway (Comai et al., 1982), the indole-3-pyruvic acid (IPA) pathway (Costacurta et al., 1995), the IAN pathway (Kobayashi et al., 1993), and the tryptamine pathway (Hartmann et al., 1983).
Thus IAN has been implicated in both Trp-dependent and Trp-independent pathways, but definitive evidence that IAN serves as an IAA precursor in vivo is lacking.

The IPA pathway has been suggested to operate in all plants. There are three steps in this pathway that convert Trp to IAA: Trp to IPA, IPA to indole-3-acetic acid (IAALD), and IAALD to IAA (Costacurta, 1995) (Figure 2). The enzymes which catalyze these three reactions are Trp aminotransferase, IPA decarboxylase (IPDC), and IAALD oxidase, respectively. Unfortunately, few data are available to support the idea that the IPA pathway is functional in plants. Two problems are related to the putative function of this pathway in vivo (Kawaguchi and Syono, 1996). The first is the low substrate-specificity and high Km for L-Trp of Trp aminotransferase, the enzyme that catalyzes the first and rate-limiting step of the pathway. The second is the high instability of IPA. Because of the instability of IPA, no IPDC activity has been established in plants. However, the genes encoding IPDC have been isolated from several microbes, e.g. *Azospirillum brasilense* (Costacurta et al., 1994), *Erwinia herbicola* (Brandl et al. 1996), and *Enterobacter cloacae* (Koga et al., 1991).

IAALD oxidase belongs to the aldehyde oxidase (AO) family, which is believed to be involved in both IAA (Figure 2) and abscisic acid biosynthesis in plants (Koshiha et al., 1996; Leydecker et al., 1995). Based on this fact, biochemists have been attempting to purify plant AOs and establish their substrate specificity for IAALD. Using an activity gel assay, two AOs have been identified in maize using indole-3-aldehyde (IALD) as substrate (Koshiha et al., 1996). Their genes (*AO1* and *AO2*) have been cloned and their proteins purified (Koshiha et al., 1996; Sekimoto et al., 1997). One AO gene (*TAO1*) has also been cloned from tomato (Ori et al., 1997).

In Arabidopsis, the same activity gel assay revealed three proteins. The top band on the activity gel, named AO1, had increased activity with IALD in the IAA overproducing Arabidopsis *suri* mutant (Seo et al., 1998). Since staining of native gels with IAALD has not been successful, IALD is the closest substrate to analyze substrate specificity for AOs involved in IAA synthesis (Koshiha et al., 1996). Thus, AO1 is considered to be an IAALD
Figure 2: IPA route of Trp-dependent IAA biosynthesis.
oxidase candidate. Recently, two different groups, Sekimoto et al. (1998) and Hoff et al. (1998), along with the Arabidopsis genome project, have combined to identify four AO genes, named \textit{AtAO1}, 2, 3, and 4 by Sekimoto et al. (1998). \textit{AtAO1}, 2, 3, and 4 were physically mapped on chromosome 5, 3, 2, and 1, respectively (Sekimoto et al., 1998). Preliminary results from Northern blot suggested that \textit{AtAO1} might be the gene encoding the top band on the activity gel since its mRNA abundance is increased in \textit{sur1} (Sekimoto et al., 1998). However, no plant AO mutants have been reported, so these genes have not been proven to be IAALD oxidase satisfactorily.

Mutant screens for IAA biosynthesis defective mutants have been performed in Arabidopsis. As mentioned above, without an effective bioassay for IPA, no IPA resistant mutants have been isolated. One IAALD resistant mutant, Y98, has been identified by Bartel (unpublished data). In this project, two more IAALD resistant mutants, a-2 and c-2, have been isolated. Phenotypic characterization and genetic analysis of these three mutants were performed in this project. Y98 was also tested for complementation by \textit{AtAO4}.

**METHODS**

**Plant Materials and Growth Conditions**

\textit{Arabidopsis thaliana} ecotypes used were Columbia (Col), Landsberg erecta (Ler), and Wassilewskija (WS). Prior to phenotypic analysis, the \textit{iar4}, \textit{ilrl-1}, \textit{iarl-1}, and \textit{iar3-2} lines had been backcrossed to WS twice, four times, three times, and twice, respectively (B. Bartel, personal communication), and seeds analyzed were homozygous mutants from the \textit{F1}, \textit{F2}, \textit{F3}, and \textit{F4} generation, respectively. Plants were surface-sterilized with bleach solution (30% Chlorox plus 0.01% Triton X-100) and grown on PNS (plant nutrient medium with 0.5% sucrose; Haughn and Somerville, 1986) solidified with 0.6% agar alone or supplemented with 0.1 µM IAA (from 1 mM stocks in ethanol), 30 to 200 µM IAA conjugates (from 100 mM stocks in ethanol), 4 to 20 µM IAALD (from 10 mM stocks in ethanol), or 15 µg/mL kanamycin (from 25 mg/mL stock in water). IAA conjugates were
from Aldrich (Milwaukee, WI). IAALD was from Aldrich and purified by Dr. Seiichi Matsuda. Plates were sealed with gas-permeable Leukopor surgical tape (Beiersdorf Inc., Norwalk, CT) and incubated under continuous light (25 to 45 μE m⁻² sec⁻¹) with yellow filters to prevent breakdown of indolic compounds (Stasinopoulous and Hangarter, 1990). Seedlings were grown at 22°C unless otherwise indicated.

Isolation of Mutants

~60,000 ethyl methanesulfonate (EMS) mutagenized M₂ seeds (ecotype Col) were plated on PNS medium containing 7 μM IAALD at a density of ~1000 seeds per 150 mm plate. After 10 days, putative mutants with increased root length were transferred to soil and allowed to self pollinate. M₃ seeds were screened separately for resistance to 7 μM IAALD and wild-type sensitivity to 0.5 μM IAA. Two mutants, a-2 and c-2 were isolated and M₄ seeds were used in subsequent experiments. Both mutants were backcrossed to Col to remove unlinked mutations and determine dominance. They were also crossed to Y98 (isolated by Bartel, unpublished data) and each other to test allelism.

Phenotypic Analysis

For root length bioassays, plants were grown on PNS alone or containing required indolic compounds (IAA, IAA conjugates or IAALD) for 8 days. Seedlings were removed from plates to measure root lengths with a ruler.

For hypocotyl elongation assay at 28°C, plants were grown on PNS medium at 28°C with other growth conditions as described above. Hypocotyl lengths were measured with a ruler after 8 days. Plants grown at 22°C were used as controls.

For hypocotyl elongation assay in the dark, plants were grown on PNS alone or containing required indolic compounds (IAA-Ala or IAALD) under continuous light for 24 hours and then wrapped in foil to block the lights. After another 4 days of growth, hypocotyl lengths were measured.
Statistical Analysis

To test whether two sets of data are statistically different, equal number of samples from each data were compared using the Systat program to perform an independent t-test. A calculated probability of less than 0.001 is considered to be statistically different.

Mapping Mutants

For genetic mapping, iar4 (isolated by Bartel from the WS ecotype, unpublished data) was crossed to both Col and Ler and F2 seeds were collected. The iar4 mutant was mapped by plating F2 seeds from both Col and Ler out-crosses on PNS plates containing 50 µM IAA-Ala. After 7 to 8 days, no more than one quarter of seedlings with long roots (putatively homozygous at the mutated locus) compared to WS were transferred to soil for another 7 to 8 days of growth. Leaf DNA was prepared by standard methods (Celenza et al. 1995) for mapping. Two polymerase chain reaction (PCR) programs: I) 40 cycles of 15 sec at 94°C, 15 sec at 55°C, and 30 sec at 72°C; II) 40 cycles of 30 sec at 95°C, 30 sec at 55°C, and 3 min at 72°C were used to determine the ecotype of each individual F2 line at certain markers (Table II). The markers used were simple sequence length polymorphisms (SSLP) markers, which detect length difference between ecotypes (Bell et al., 1994), or cleaved amplified polymorphic sequences (CAPS) markers for their restriction enzyme site polymorphisms between ecotypes (Konieczny et al., 1993).

Y98 (from the Col ecotype) was crossed to WS and F2 seeds were collected. The Y98 mutant was mapped by plating F2 seeds from a WS out-cross on PNS plates containing 7 µM IAALD to get IAALD resistant F2 seedlings. PCR programs and type of markers used were the same as used for iar4 mapping.

Developing New Polymorphic Markers
To develop new markers, primers (see Table II) were designed from genomic DNA sequence (Col) reported by the genome project and used to PCR amplify products from WS or Ler genomic DNA prepared from leaf tissues. The PCR program used was program II (Table II). PCR products were sequenced directly (University of Texas-Houston Medical School) following PEG precipitation (Ausubel et al., 1995) to find polymorphisms among different ecotypes.

Construction of A Genomic Sub-library from BAC F3I6

DNA from BAC F3I6 was prepared using the Qiagen plasmid kit and partially digested with the restriction enzyme Sau3AI, which generates the same cohesive ends as BamHI, to give the optimal band size of 8-14 kb. Digestion products were separated on 0.8% agarose gel containing 1 mM guanosine to protect DNA from UV damage (Grundermann and Schomig 1996). DNA fragments larger than 8 kb were excised and purified using the QIAEX II Gel Extraction Kit (Qiagen), cloned into the BamHI site of binary vector pBIN19 (Bevan 1984), and transformed into E. coli strain DH5α. Transformants containing BAC DNA were white in the presence of 50 μg/mL kanamycin and X-gal, and confirmed by restriction digestion.

Activity Gel Assays

Y98, a-2, c-2 and Col seeds were grown on unsupplemented media for 7 days. Seedlings were frozen in liquid nitrogen, ground into powder using a mortar and pestle, and dissolved in 0.1 M Tris (pH 6.8) and 20% glycerol to extract total proteins. Proteins were then separated on a 30% acrylamide/0.8% bisacrylamide native gel system at 4°C (Laemmli 1970). Activity staining was performed as described by Koshiba et al. (1996). 1 mM IALD was used as substrate.
Complementation Test of Y98 by *AtAO4*

An 8 kb BamHI fragment containing the *AtAO4* gene (5.3 kb) including 2 kb 5’ and 0.7 kb 3’ sequence was prepared from bacterial artificial chromosome (BAC) T1G11 (from the Arabidopsis Biological Research Center, Ohio State University) and cloned into pBluescript II KS(+) using Fast-Link ligase (Epicentre Technologies). This BamHI fragment was then cloned into the binary vector pBIN19 (Bevan, 1984) and transformed into DH5α. Transformants that contained *AtAO4* were chosen for plasmid preparation and this DNA was transformed into *Agrobacterium tumefaciens* GV3101 by electroporation (Ausubel et al., 1995). Transformed *Agrobacterium* was used to infect Y98 plants by infiltration as described (Clough and Bent, 1998). Seeds were screened by growing for 2 weeks on PN containing 15 μg/mL kanamycin. Transformants were identified by their resistance to kanamycin and transferred to soil to produce seeds. Seeds from transformants were plated on PNS medium containing 7 μM IAALD to perform root length bioassays to test complementation.

Sequencing of *AtAO4* in Y98

The coding region of *AtAO4* was amplified from genomic DNA of Y98 using a PCR program II (Table II). PCR products were sequenced directly (Lone Star Labs. Inc., Houston, TX) with each of the oligos used for PCR. Oligonucleotides used were the following pairs: 5’-GCAGCTAACTGTGTCCTATTGATTG-3’ and 5’-CTATCTTTGCCAGGTTTGTGGTG-CC3’; 5’-GGATCAGAGCAAGGTATCATCATTGGAGGC-3’ and 5’-GCTGCTTGAGCCTTTTCACAGCATTG-3’; 5’-GAAGAGTTGGAGGTGGCTTGGTGG-3’ and 5’-GAGCTGATAGGTCCACAGATTGAGC-3’; 5’-GTGACATGGGACA-TGCCATTTCAAC-3’ and 5’-CCGGTTAAAGACTATTTCACTTTTCG-3’; 5’-CACCCT-GTTGGTGAAGCCATTATC-3’ and 5’-CACATGCTGTCGCAACCTGAAATC-3’.
RESULTS

Part I. iar4

iar4 Is an IAA-Ala Resistant Mutant

iar4 was originally isolated as an IAA-Ala resistant mutant (Bartel, unpublished). To compare the phenotype of iar4 in the presence of IAA conjugates to those of other mutants, root length bioassays were performed on iar4, WS (wild-type), iar1-1, iar3-2, and ilrl-1 lines (see Methods).

Figure 3 shows the root lengths of the each on different media. When grown on unsupplemented medium, ilrl-1, iar1-1, and iar3-2 seedlings have similar root growth to WS, but iar4 seedlings grow only as half long as WS. Thus iar4 has a defect in root growth. In the presence of 0.1 μM IAA, all 4 mutant lines and wild type were similarly inhibited (Figure 3). Like iar1 and iar3, iar4 is clearly resistant to 30 μM IAA-Ala since its roots grew about 2 fold longer than WS, but it is not resistant to 30 μM IAA-Leu. iar4 is also resistant to 30 μM IAA-Phe, as are iar1 and ilrl. The partial resistance to IAA-Phe of iar1, iar4, and ilrl suggests that there might be genetic redundancy in converting IAA-Phe to IAA, therefore providing the reason that it is difficult to isolate IAA-Phe resistant mutants directly from EMS-mutagenized seeds (Bartel, personal communication).

iar4 Hypocotyl Elongation Assays

Gray et al. (1998) reported that when grown at 28°C, Arabidopsis seedlings have elongated hypocotyls, and that this elongation is mediated by elevated levels of endogenous IAA. However, it is unknown how plants produce higher level of IAA at 28°C. To investigate whether hydrolysis is involved, hypocotyl elongation assays were performed (see Methods) on all 4 IAA conjugate resistant mutants.

Figure 4 shows hypocotyl growth of all 4 mutants and wild-type at 28°C and 22°C. Each line shows about 2 fold more elongation at high temperature. These results imply that
Figure 3: Root lengths of IAA conjugate mutants on various media after 8 days at 22°C under yellow-filtered light. At least 9 seedlings of each line were measured. Error bars indicate standard deviations of the mean. Asterisks indicate that the data are significantly different from wild type under the same condition (t-test, P<0.001).
Figure 4: Hypocotyl elongation of IAA conjugate mutants at different temperatures after 8 days under yellow-filtered light. At least 9 seedlings of each line were measured. Error bars indicate standard deviations of the mean. This experiment has not been repeated.
resistance to IAA conjugates does not affect the increased level of IAA at high temperature, which causes hypocotyl elongation.

It has also been observed that high concentrations of IAA inhibit hypocotyl elongation in the dark (Bartel and Lasswell, unpublished data). Again the mechanism involved is not clear. To gain more information, hypocotyl elongation assays in the dark (see Methods) were performed on all four IAA conjugate mutants.

Figure 5 shows the percentage of hypocotyl elongation in the dark compared with the control. In the presence of 200 μM IAA-Ala, there is no major difference between WS and the mutants. At 100 μM IAA-Ala, all four IAA conjugate mutants show various degrees of resistance to IAA-Ala compared to wild type. Although on root length assay, ilr1-1 does not show resistance to 30 μM IAA-Ala (Figure 3), its resistance in the dark on hypocotyl elongation assay is not considered an aberration because the ILR1 protein hydrolyzes IAA-Ala in vitro (Bartel and Fink, 1995), although not as efficiently as IAA-Phe or IAA-Leu. These data indicate that hypocotyl elongation in the dark is affected in IAA conjugate resistant mutants, supporting the observation that higher concentrations of IAA inhibit hypocotyl elongation in the dark (Lasswell and Bartel, unpublished data).

Genetic Mapping of iar4

The map position of iar4 mutant was determined by recombination mapping (see Methods). Initial mapping data showed that iar4 is located between SSLP marker nga128 (Bell et al., 1994) and CAPS marker m235 (Hardtke, unpublished data) on chromosome 1 (Table I). Since the Arabidopsis sequencing project provides part of the sequences between these two markers, new CAPS markers were developed (Tables I, II) using these sequences (see Methods) to narrow down the mutant position. Mapping data from a Ler cross showed that iar4 is south of F3I6-6+7 and north of T7N9 (Table I). Normally, one cM in Arabidopsis is about 100 to 200 kb (Koornneef, 1994). Since only one north recombinant was detected at F3I6-6+7, iar4 is supposed to be closely south of this marker. If this is true,
Figure 5: Hypocotyl elongation of IAA conjugate mutants in the dark at 22°C. At least 9 seedlings of each line were measured. Data were normalized as percentage elongation compared with the unsupplemented control. Error bars indicate standard deviations of the mean. This experiment has not been repeated.
Figure 6: Positions of Markers used for mapping *iar4*. Position of markers in cM are shown in parentheses [from the updated version (11/28/1998) of the recombinant inbred map (http://nasc.nott.ac.uk/new_ri_map.html)]. BAC names are underlined. Newly-developed markers are indicated at their locations on respective BACs. Size of BAC: T23E23, 99 kb; F3I6, 122 kb; F21J9, 99 kb. Also shown is BAC T1G11 on which *AtAO4* is located and marker nga63 which is used for mapping Y98.

Table I: *iar4* Mapping data

<table>
<thead>
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<th>Markers</th>
<th>Position (cM from North)</th>
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<tr>
<td>nga59</td>
<td>~2.4</td>
<td></td>
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<tr>
<td>m235</td>
<td>~32</td>
<td></td>
</tr>
<tr>
<td>T23E23</td>
<td>~90 kb north of F3I6-6+7</td>
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<td>F3I6-2+3</td>
<td>~6 kb north of F21J9-9+10</td>
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<td>F21J9-9+10</td>
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<td></td>
</tr>
<tr>
<td>F21J9-1+2</td>
<td>~70 kb south of F21J9-9+10</td>
<td>0/542</td>
</tr>
<tr>
<td>T7N9</td>
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<tr>
<td>nga392</td>
<td>very close to T7N9</td>
<td>4/104</td>
</tr>
<tr>
<td>nga280</td>
<td>~80</td>
<td></td>
</tr>
<tr>
<td>nga128</td>
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* Data are presented as number of recombinations detected out of number of chromosomes scored.
<table>
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<tr>
<th>Marker</th>
<th>Type</th>
<th>Primers</th>
<th>*Polymorphism</th>
<th>**PCR program</th>
<th>% gel used</th>
<th>Reference</th>
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<tr>
<td>nga59</td>
<td>SSLP</td>
<td>5'-GCATCTGGGTACACTCGCC-3' 5'-TTAATACATTAGCCAGAGCCG-3'</td>
<td>Col, 0.111; Ler, 0.115; WS, 0.083</td>
<td>I</td>
<td>4</td>
<td>Bell and Ecker, 1994</td>
</tr>
<tr>
<td>nga63</td>
<td>SSLP</td>
<td>5'-AAACCGATTAGGCCAGAGCG-3' 5'-ACCAAGGCTACACTCGCCAC-3'</td>
<td>Col, 0.111; WS, 0.083</td>
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<td>4</td>
<td>Bell and Ecker, 1994</td>
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<tr>
<td>m235</td>
<td>CAPS</td>
<td>5'-GAATCTGTTTCGCTAAGCG-3' 5'-GTCCACAAACTAATTCAGCGCC-3'</td>
<td>HindIII: Ler, 0 (0.534); WS, 1 (0.309, 0.225)</td>
<td>I</td>
<td>2</td>
<td>C. Hardie and T. Berleth, unpublished</td>
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<tr>
<td>T23E23</td>
<td>CAPS</td>
<td>5'-CATTACAGTCTTATGAGAGGATCG-3' 5'-GACACAGATCTTATGAGAGGATCG-3'</td>
<td>BsrBI: Col=Ler, 0 (1.17); WS, 1 (0.37, 0.8)</td>
<td>II</td>
<td>2</td>
<td>This work</td>
</tr>
<tr>
<td>F316-6+7</td>
<td>CAPS</td>
<td>5'-GAATTTACGTGCAGTGTGGTTG-3' 5'-GATACACTCTCTCTGAGTGGAGCT-3'</td>
<td>Avall: Col=Ler, 0 (1.02); WS, 1 (0.43, 0.59)</td>
<td>II</td>
<td>2</td>
<td>This work</td>
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<tr>
<td>F316-2+3</td>
<td>CAPS</td>
<td>5'-CACTACAGTCTTATGAGAGGATCG-3' 5'-GACACAGATCTTATGAGAGGATCG-3'</td>
<td>HindIII: Ler, 1 (0.27, 0.05); WS, 0 (0.32)</td>
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<td>2</td>
<td>This work</td>
</tr>
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<td>SspI: Col, 1 (0.3, 1.05); WS, 0 (1.35)</td>
<td>II</td>
<td>2</td>
<td>This work</td>
</tr>
<tr>
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<td>CAPS</td>
<td>5'-GTACGTGCTCAGGGAAGCAGT-3' 5'-GATACACTCTCTCTGAGTGGAGCT-3'</td>
<td>NsiI: Col=Ler, 1 (0.48, 1.32); WS, 0 (1.8)</td>
<td>II</td>
<td>2</td>
<td>This work</td>
</tr>
<tr>
<td>T7N9</td>
<td>CAPS</td>
<td>5'-GTAGCCACCGAAGATTAGTG-3' 5'-GGTGGTGAA-3'</td>
<td>HaeIII: Col=Ler, 1 (0.13, 0.73); WS, 0 (0.86)</td>
<td>II</td>
<td>2</td>
<td>This work</td>
</tr>
<tr>
<td>nga280</td>
<td>SSLP</td>
<td>5'-CTGATACGCGGACAATAGTG-3' 5'-GCTCCATTTTGAGGTGAC-3'</td>
<td>Col, 0.105; WS, 0.085</td>
<td>I</td>
<td>4</td>
<td>Bell and Ecker, 1994</td>
</tr>
<tr>
<td>nga128</td>
<td>SSLP</td>
<td>5'-GGTGGGTAGTGAGTGCG-3' 5'-ACTCTTTACCCCGAGGAGG-3'</td>
<td>Ler, 0.19; WS, 0.172</td>
<td>I</td>
<td>4</td>
<td>Bell and Ecker, 1994</td>
</tr>
</tbody>
</table>

*Polymorphisms for SSLP markers are listed as size of fragments (kb) for each ecotype. For CAPS markers, it is listed as restriction enzymes used, number of recognition sites, and size of fragments (kb) after digestion in parentheses for each ecotype. **PCR program I: 40 cycles of 15 sec at 94°C, 15 sec at 55°C, and 30 sec at 72°C. Program II: 40 cycles of 30 sec at 95°C, 30 sec at 55°C, and 3 min at 72°C.
at least one recombinant was expected at F21J9-1+2, which is about 200 kb south of F3I6-6+7 (Figure 6). However, no recombinants were detected, suggesting that recombination frequency is suppressed around that area in the cross between WS and Ler. Therefore, I switched to a Col cross.

The mapping data from the Col cross show that iar4 is north of F21J9-9+10, which is about 120 kb south of F3I6-6+7 (Figure 6). There were 2 recombinations out of 240 chromosomes at F21J9-9+10 (Table 1), suggesting that iar4 is north of this locus. There were no recombinants at F3I6-6+7, suggesting that iar4 is very close to this marker so that 240 chromosomes would not be enough to guarantee one recombination. However, combining the data from both Ler and Col crosses, iar4 should be located between F3I6-6+7 and F21J9-9+10, a span of about 100 kb, which will be a decent size to use for complementation testing to clone the gene.

The mapping data of iar4 suggest that it is located on BAC F3I6. Therefore, F3I6 was subcloned into the binary vector pBIN19 to construct a library for future complementation tests (see Methods). Subclones were aligned based on their restriction digestion pattern and will be confirmed by sequencing. Currently about 95% of the F3I6 insert is covered on 16 subclones.

**Part II: IAALD resistant mutants**

**Isolation and Phenotypic Characterization of IAALD Resistant Mutants**

To isolate more IAA biosynthesis mutants, about 60,000 M2 EMS-mutagenized Col seeds from 10 pools were screened for resistance to 10 μM IAALD (see Methods). 40 M2 lines were identified and 21 of them produced seeds. M3 seeds of these 21 lines were screened for sensitivity to 0.5 μM IAA. Among them 6 were sensitive, suggesting they are truly resistant to IAALD. Retesting these 6 lines in the presence of 7 μM IAALD showed
two mutants, a-2 and c-2, had consistently increased root growth compared to wild type. It is not yet known whether these two mutants are alleles of each other or of Y98.

To characterize the phenotype of the mutants, Y98, a-2, and c-2 seeds were grown in the presence of 5 or 10 μM IAALD or 0.1 μM IAA to perform root length assay (see Methods). All three mutants are sensitive to 0.1 μM IAA, as their root growths were similarly inhibited (Figure 7). When grown in the presence of 5 or 10 μM IAALD, their percentage root growths were at least as twice that of wild-type. Therefore, all three mutants are resistant to IAALD, and c-2 is the strongest one.

Hypocotyl elongation assays at different temperatures were also performed on all the mutants (see Methods). When grown at normal temperature (22°C), all three mutants had shorter hypocotyls than wild-type (Figure 8) but the difference is not statistically significant (t-test, P>0.001). At 28°C, like wild-type, all three mutants showed increased hypocotyl growth (Figure 8), suggesting that they were able to increase endogenous IAA levels. Moreover, although all the mutants appeared to have a larger degree of increased hypocotyl growth than wild-type, their average hypocotyl lengths were either shorter than or the same as that of wild-type (Figure 8). It is not known how IAA mediates hypocotyl growth, but our data indicate that these IAALD resistant mutants are competent to increase hypocotyl elongation at high temperature, thus implying that they are able to produce higher level of endogenous IAA.

Hypocotyl elongation assays in the dark were also performed on the IAALD resistant mutants (see Methods). Mutant seeds along with wild-type were grown in the presence of 0 or 20 μM IAALD. When grown on unsupplemented media, all three mutants had normal hypocotyl elongation in the dark as the wild type (Figure 9). At 20 μM IAALD, hypocotyl growth seems to be affected very little as wild type still showed about 80% elongation compared with the unsupplemented control. Y98 showed similarly reduced hypocotyl elongation to the wild type while the other two mutants displayed different degrees of resistance with c-2 almost unaffected (Figure 9). Therefore, we suggest that further
Figure 7: Percentage root growth of IAALD resistant mutants on different media after 8 days at 22°C under yellow-filtered light. At least 9 seedlings of each line were measured. Data were normalized as percentage elongation compared with the unsupplemented control. Error bars indicate standard deviations of the mean. Asterisks labeled on top of each column indicate that the data are significantly different from wild type under the same condition (t-test, P<0.001). This experiment has not been repeated.
Figure 8: Hypocotyl elongation of IAALD resistant mutants at different temperatures after 8 days under yellow-filtered light. At least 9 seedlings of each line were measured. Error bars indicate standard deviations of the mean. This experiment has not been repeated.
Figure 9: Hypocotyl elongation of IAALD resistant mutants in the dark at 22°C. At least 9 seedlings of each line were measured. Error bars indicate standard deviations of the mean. This experiment has not been repeated.
investigation at higher concentration of IAALD could be more useful to gain information about how hypocotyl growth of these mutants respond to IAALD in the dark.

Activity gel assays were also performed on three mutants (see Methods). As reported previously (Seo et al., 1998), three bands were detected in the wild type using IALD as a substrate. No difference was observed between the wild type and the three mutants in the intensity of bands. Since four AtAO genes have been reported to exist in Arabidopsis (Sekimoto et al., 1998), it is unknown why only three bands were detected using IALD as substrate in wild-type. The reason could be that the missing AO on the activity gel is specific to IAALD or that the in vitro conditions used are not optimal enough to detect that protein. Therefore, if the gene defective in the three mutants encodes the missing AO, the activity gel will not be able to distinguish between the wild-type and mutants. However, it is also possible that the genes defective in those mutants do not encode AOs.

Complementation Test of Y98 by AtAO4

The Y98 mutant was isolated from the Col ecotype. F2 seeds from an outcross to WS were used for mapping. Preliminary mapping data suggested that Y98 is located between SSLP markers nga59 and nga63 (Bell et al., 1994) on the top of chromosome 1 (Table III). Since AtAO4 is found on BAC T1G11 in the same region (Figure 6; Sekimoto et al., 1998), it is possible that AtAO4 is the gene defective in Y98 mutant. Therefore, I tested complementation of Y98 by AtAO4.

The AtAO4 gene (5.3 kb) and its promoter region was cloned into the binary vector pBIN19 (Bevan, 1984) and transformed into Y98 (see Methods). Two transformed plants (T1) were identified and transferred to soil to produce seeds. T2 seeds were grown in the presence of IAALD to test for complementation.

Figure 10 shows the distribution of root length of the progeny from two transformed plants, Y98(AO4)-1 and Y98(AO4)-2. Since both transformed plants were heterozygous for the transgene, if AtAO4 is able to complement Y98, 3/4 of the T2 progeny should have the
Table III: Y98 mapping data

<table>
<thead>
<tr>
<th></th>
<th>Position (cM from north)</th>
<th>*Mapping data</th>
</tr>
</thead>
<tbody>
<tr>
<td>nga59</td>
<td>~2.4</td>
<td>7/40</td>
</tr>
<tr>
<td>nga63</td>
<td>~10</td>
<td>9/40</td>
</tr>
</tbody>
</table>

* Data are presented as number of recombinations detected out of number of chromosomes scored.
Figure 10: Distribution of root length of the progeny from Y98(AO4)-1 and Y98(AO4)-2 in the presence of 7 μM IAALD after 8 days at 22°C under yellow-filtered light. Roots were measured to the nearest 0.5 mm and grouped in 2 mm intervals. Number of seedlings of each group is indicated.
same sensitivity to IAALD as wild-type (Col). The results showed that about 3/4 (17 out of 20 for Y98(AO4)-1; 14 out of 20 for Y98(AO4)-2) of the progeny of both transformed plants had the same range of root length range as Col, suggesting that AtAO4 complements Y98.

To confirm that AtAO4 is the gene defective in the Y98 mutant, primers were designed to amplify the coding region of AtAO4 in Y98 by PCR (see Methods). PCR products were sent for sequencing. However, no mutation was found on the coding region (including the intron-exon boundaries) of AtAO4 from Y98 (data not shown).

Discussion

**iar4 Is An IAA-Ala Resistant Mutant**

Hydrolysis of IAA conjugates plays a very important role in IAA metabolism. Study of mutants resistant to IAA conjugates enables us to learn valuable information about the mechanism of hydrolysis. In this project, we characterized an IAA-Ala resistant mutant from Arabidopsis thaliana, iar4 (isolated by Bartel, unpublished data) and used recombination mapping to locate the mutation.

When grown on 0.1 μM IAA, root growth of iar4 is inhibited, as are other IAA conjugate mutants and wild type (Figure 4), indicating that iar4 is sensitive to IAA. Like iar1-1 and iar3-2, iar4 shows root length at least as twice long as wild type in the presence of 30 μM IAA-Ala. iar4 is also resistant to 30 μM IAA-Phe, although not as strong as its resistance to IAA-Ala, but sensitive to 30 μM IAA-Leu, suggesting IAR4 may also be involved in converting IAA-Phe to IAA. Interestingly, roots of iar4 only grow about half long as those of wild type and other IAA conjugate mutants studied on unsupplemented media (Figure 3). Thus iar4 is unique among the IAA conjugate mutants analyzed to date in that it has a phenotype in the absence of exogenous conjugates. Although iar4 mutant plants used in this study had been backcrossed twice to the wild type, further backcrosses
will be necessary to ensure this root elongation defect is not due to an unrelated mutation. Based upon these data, we conclude that *iar4* is an IAA-Ala resistant mutant.

To locate *iar4*, we performed recombination mapping on F₂ progeny of both Col and Ler outcrosses. Our mapping data indicate that *iar4* is located between markers F3I6-6+7 and F21J9-9+10 (Table I) on the top of chromosome I. According to the Arabidopsis sequencing project, the distance between these two markers is about 120 kb (Figure 7), which will be convenient for complementation tests.

What does *IAR4* do in plants? Like *IAR3*, *IAR4* could encode an IAA-Ala hydrolase. Also *IAR4* could encode an IAA-Ala transporter. Although until now no IAA conjugate transporter has been identified, we believe that IAA conjugate transporters exist because of following observations. The sequences of several IAA conjugate hydrolases have endoplasmic reticulum (ER) retention signals, KDEL or derivatives (Davies et al., 1999), suggesting that they are retained in the lumen of the ER. Since IAA conjugates can not freely enter cells, transporters are required. The sequence of *IAR1* suggests that it may encode a protein with 8 transmembrane domains (Lasswell and Bartel, unpublished data), which could be a putative transporter. Annotation of BAC F3I6 by the genome project has revealed eight genes similar or identical to genes of known function and several other putative genes with no apparent homologies to current known genes (Figure 11). However, none of the eight are similar to *IAR1* or *IAR3*. Therefore, *IAR4* could encode a protein with a function other than a hydrolase or a transporter, such as a transcription factor or other regulatory protein which regulates hydrolases or transporters.

**IAA Biosynthesis Mutants**

It is believed that both Trp-dependent and Trp-independent IAA synthetic pathways exist in plants. We performed a genetic screen for new IAALD resistant mutants. Two mutants, a-2 and c-2, were isolated. They, along with Y98 (Bartel, unpublished data), showed increased root growth compared with wild type in the presence of 5 μM or 10 μM IAALD,
Figure 11: Potential coding regions of BAC F3I6. Size of the BAC is shown as numbers of base pairs. Restriction sites of BamHI and EcoRI are shown as bars. Potential coding regions are shown as boxes (exons) connected by dashed lines (introns). Question marks are potential genes of unknown functions.
while root elongation of all three mutants were similarly inhibited as wild type by IAA (Figure 7). It is not known whether they belong to the same complementation group. The isolation of IAALD resistant mutants with normal IAA sensitivity suggests that wild-type plants are able to convert IAALD to IAA.

Normanly et al. (1993) reported that two Trp synthesis mutants, trp2 and trp3, accumulated high level of IAA, suggesting the existence of a Trp-independent IAA biosynthetic pathway. Moreover, they also accumulated high level of IAN, a potential precursor of IAA. Four nitrilase (NIT) genes, which encode the enzymes converting IAN to IAA, have been isolated from Arabidopsis (Bartel and Fink, 1994). Mutants of NIT1 showed increased root growth compared with wild type on 30 μM IAN. Therefore, Arabidopsis is able to utilize IAN as an IAA precursor, and IAN seems to be involved in the Trp-independent pathway. However, using isotope-labeling coupled with GC-MS, Normanly et al. (1993) found that IAN was more likely to be an intermediate in the Trp-dependent pathway. Investigation of multiple mutants of AO, NIT, and Trp synthesis should provide more information.

**Hypocotyl Elongation Is Affected by IAA**

Gray et al. (1998) reported that at 28°C, Arabidopsis had increased level of endogenous IAA, which led to increased hypocotyl growth. It is not clear how IAA production is increased. We performed a similar assay on both IAA conjugate resistant mutants and IAALD resistant mutants. At 28°C, both of them showed similar increased hypocotyl growth to wild type (Figure 4 and Figure 8). It is possible that neither hydrolysis of IAA conjugates nor IAALD oxidation is involved in producing of high level of IAA. However, it is also possible that both of them are involved and deficiency in one of them is compensated by the other one. Study of IAA conjugate resistant and IAALD resistant double mutants will be more informative.
In the dark, hypocotyl elongation is inhibited by high concentration of IAA (Lasswell and Bartel, unpublished data). We studied hypocotyl elongation of both IAA conjugate resistant and IAALD resistant mutants in the dark. At 100 μM IAA-Ala, iar4, ilrl, iar1, and iar3 all showed increased hypocotyl growth compared with wild type while at 200 μM concentration, all four mutants had similarly inhibited hypocotyl growth as wild type did (Figure 5). For IAALD resistant mutants, 20 μM IAALD appeared to have little affect on their hypocotyl growth. Both wild type and mutants showed about 80% of hypocotyl growth compared with the unsupplemented control (Figure 9). However, two mutants, a-2 and c-2, showed a little degree of resistance with c-2 almost unaffected (Figure 9). Therefore, using a higher concentration of IAALD might be able to separate the behaviors between wild type and mutants. Our data support that a high concentration of IAA inhibits hypocotyl elongation in the dark. In addition, the resistance of the mutants to the inhibitory effects of IAA precursors on both root and hypocotyl elongation suggests that the affected gene products normally function in these tissues.

Is AtAO4 the Gene Defective in Y98?

Our preliminary mapping data of Y98 showed the mutant was located in the same region as AtAO4 (Table III), a cloned Arabidopsis AO gene suggested to be involved in IAA biosynthesis. Therefore, we performed a complementation test of Y98 by AtAO4. Two transformed plants were isolated, and their T2 progeny were used for root length assays. Data from both lines showed that about 3/4 of T2 seedlings had similarly inhibited root growth as wild type (Figure 10), indicating that AtAO4 complements the Y98 mutant. To confirm that AtAO4 is the gene defective in Y98, we sequenced all the coding regions of AtAO4 from the mutant. However, no mutations were found. There are several possibilities: 1) the mutation is on the promoter of the gene; 2) the mutation in Y98 is a methylation mutation, where increased AtAO4 methylation results in decreased transcription; 3) the mutation is in the intron region which has not been sequenced. We believe that AtAO4 is the
gene defective in the Y98 mutant and aldehyde oxidase is involved in IAA synthesis in Arabidopsis.
References:


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interactions with the hyg-1 hypocotyl elongation and axr1 auxin-resistant mutants. *Plant Mol. Biol.* **27**, 1071-1083.


