**IAR4**, a Gene Required for Auxin Conjugate Sensitivity in Arabidopsis, Encodes a Pyruvate Dehydrogenase E1α Homolog

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The formation and hydrolysis of indole-3-acetic acid (IAA) conjugates represent a potentially important means for plants to regulate IAA levels and thereby auxin responses. The identification and characterization of mutants defective in these processes is advancing the understanding of auxin regulation and response. Here we report the isolation and characterization of the Arabidopsis *iar4* mutant, which has reduced sensitivity to several IAA-amino acid conjugates. *iar4* is less sensitive to a synthetic auxin and low concentrations of an ethylene precursor but responds to free IAA and other hormones tested similarly to wild type. The gene defective in *iar4* encodes a homolog of the E1α-subunit of mitochondrial pyruvate dehydrogenase, which converts pyruvate to acetyl-coenzyme A. We did not detect glycolysis or Krebs-cycle-related defects in the *iar4* mutant, and a T-DNA insertion in the *IAR4* coding sequence conferred similar phenotypes as the originally identified missense allele. In contrast, we found that disruption of the previously described mitochondrial pyruvate dehydrogenase subunit does not alter IAA-Ala responsiveness or confer any obvious phenotypes. It is possible that *IAR4* acts in the conversion of indole-3-pyruvate to indole-3-acetyl-coenzyme A, which is a potential precursor of IAA and IAA conjugates.

Auxins affect virtually every aspect of plant development, including phototropism, gravitropism, cell expansion, apical dominance, root growth, fruit development, vascular development, and senescence (Davies, 1995). By understanding how plants regulate levels of free indole-3-acetic acid (IAA), the active form of the most abundant naturally occurring auxin, we can gain insight into how plants develop and respond to environmental stimuli.

The IAA in Arabidopsis is found in three basic forms. The free acid is the active form of IAA but constitutes only a small fraction of the total IAA in Arabidopsis. The majority of IAA is found conjugated to peptides or amino acids via amide linkages or to sugars via ester linkages (Bartel et al., 2001; Ljung et al., 2002). This high proportion of conjugates suggests that auxin homeostasis may be regulated through formation and hydrolysis of conjugates in addition to regulation through de novo synthesis, transport, degradation, and interconversion between IAA and indole-3-butyric acid (Bartel et al., 2001; Ljung et al., 2002). IAA conjugates may function as storage, inactivation, or transport forms of IAA (Hangarter et al., 1980; Nowacki and Bandurski, 1980; Slovin, 1997) and some conjugates may have roles independent of hydrolysis (Hangarter et al., 1980; Magnus et al., 1992a).

The amide conjugates IAA-Asp, IAA-Glu, IAA-Ala, and IAA-Leu have been identified in Arabidopsis seedlings (Tam et al., 2000; Kowalczyk and Sandberg, 2001), and an IAA-peptide is abundant in Arabidopsis seeds (Walz et al., 2002). Certain endogenous IAA conjugates can elicit auxin responses in bioassays (Hangarter et al., 1980; Hangarter and Good, 1981; Bialek et al., 1983; Magnus et al., 1992b; Davies et al., 1999; LeClere et al., 2002), and conjugate activity often correlates with hydrolysis (Bialek et al., 1983; LeClere et al., 2002). For example, many IAA-amino acid conjugates inhibit Arabidopsis root elongation like IAA, and this bioactivity correlates with in vitro hydrolysis by heterologously expressed amidohydrolases (LeClere et al., 2002).

To enhance our understanding of the function and regulation of auxin conjugates, we have conducted screens for mutants that remain sensitive to free IAA but have reduced sensitivity to IAA conjugates. If conjugates are IAA precursors, then these conjugate resistant mutants may identify genes necessary for conjugate hydrolysis or uptake. If conjugates have additional roles, it also may be possible to genetically separate conjugate functions from those of IAA. The analysis of IAA-conjugate resistant mutants to date suggests that IAA conjugates with auxin activity act via their hydrolysis to release free IAA. Reported IAA-conjugate resistant mutants are defective in genes encoding IAA-conjugate hydrolases (*ilr1*; Bartel and Fink, 1995; and *iar3*; Davies et al., 1999) or genes predicted to affect the transport of cofactors necessary.

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for this hydrolysis (iar1 and ilr2; Lasswell et al., 2000; Magidin et al., 2003). In this work we describe the isolation and characterization of a new IAA-conjugate-resistant mutant, iar4. The gene defective in this mutant encodes a homolog of the mitochondrial pyruvate dehydrogenase (PDH) E1α-subunit.

RESULTS
Isolation and Characterization of the iar4 Mutant

The ability of certain IAA-amino acids to inhibit Arabidopsis root elongation provides a convenient bioassay to screen for mutants disrupted in conjugate perception (Bartel and Fink, 1995; Davies et al., 1999; Lasswell et al., 2000; Magidin et al., 2003). We isolated iar4-1 from ethylmethane sulfonate mutagenized Arabidopsis as an individual less sensitive than wild type to root elongation inhibition by IAA-Ala. The IAA-Ala resistance of iar4-1 is recessive (data not shown), suggesting that it is a loss-of-function allele. To explore the specificity of the iar4 conjugate response defects, we assayed iar4 root elongation on several conjugates. iar4-1 is resistant to IAA-Álα and is slightly resistant to IAA-Gly, IAA-Leu, IAA-Met, and IAA-Phe. In contrast, iar4-1 responds like wild type to the inhibitory effects of IAA-Glu, IAA-Asn, IAA-Gln, and IAA-Tyr on root elongation (Fig. 1).

We tested the iar4 response to several auxins and other phytohormones to explore the specificity of IAR4 in auxin metabolism or signaling. Because iar4-1 has a short root on unsupplemented media (Fig. 1), we compared root growth of iar4 to wild type over a range of hormone concentrations to gain a clearer picture of the ability of iar4 to perceive and respond to these compounds. To examine whether iar4 is defective in auxin responses in general, the endogenous auxins IAA and indole-3-butyric acid and the synthetic auxins 2,4-dichlorophenoxyacetic acid (2,4-D) and 1-naphthaleneacetic acid were tested. iar4 is less sensitive than wild type to the inhibition of root elongation caused by certain concentrations of 2,4-D. In contrast, iar4 responds more similarly to wild type to IAA, indole-3-butyric acid, and naphthaleneacetic acid. However, the fact that iar4 has a short root on unsupplemented medium may be obscuring any slight reduction in sensitivity to these other auxins (Fig. 2, A–D).
We also tested other phytohormones and found that iar4-1 roots respond similarly to wild type to the inhibitory effects of abscisic acid (ABA), the cytokinin benzyladenine, and the brassinosteroid brassinolide but may be slightly resistant to the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC; Fig. 2, E–H). Dark-grown iar4-1 hypocotyl elongation, however, is inhibited normally by higher concentrations of ACC (data not shown), unlike some of the previously characterized ethylene-resistant mutants (Roman et al., 1995). We conclude that IAR4 is unlikely to be involved in general hormone responses but seems to be defective in an auxin-related process.

Many plants hydrolyze conjugates during germination, and this hydrolysis is thought to supply developing seedlings with IAA (Epstein et al., 1980; Bialek and Cohen, 1992; Ljung et al., 2001; Rampey et al., 2004). Because iar4-1 is resistant to IAA-Ala, and because 8-d-old iar4-1 seedlings have shorter roots than wild type on unsupplemented medium, we examined iar4-1 germination rates. As shown in Figure 3A, the mutant germinates at the same time as wild type, and the iar4-1 defect in root elongation is not a reflection of delayed germination but persists throughout early development. The resistance to IAA-Ala is also seen throughout early development and is not the result of faster germination on the conjugate-containing medium.

Growth of Arabidopsis seedlings at high temperature (28°C) increases endogenous IAA levels (Gray et al., 1998) and can increase hypocotyl (Gray et al., 1998) and root elongation (Rogg et al., 2001). As shown in Figure 3, B and C, the difference between wild-type and iar4-1 root length on hormone-free medium is reduced in seedlings grown at 28°C in the light compared to those grown at 22°C, regardless of whether the medium is supplemented with Suc. This finding is consistent with the iar4 defect resulting from decreased endogenous auxin concentrations or a change in auxin sensitivity. However, the short root phenotype is not rescued at 22°C by growth under yellow filters (Fig. 3C), which slows the breakdown of indolic compounds (Stasinopoulos and Hangarter, 1990).

To explore whether iar4-1 might be a temperature sensitive allele and to clarify the results at 22°C that were complicated by the short root on unsupplemented medium, we measured the sensitivity of wild type and iar4 to several hormones at 28°C. Like at 22°C, we found that iar4 remains less sensitive than wild type to root inhibition by IAA-Ala and 2,4-D at 28°C and responds like wild type to other hormones tested (Fig. 3D).

**IAR4 Encodes a PDH E1α Homolog**

We identified the gene defective in iar4-1 using a map-based positional cloning strategy. By analysis

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Figure 3. iar4-1 root growth at 22°C versus 28°C. A, Seedlings were grown on vertical plates either lacking hormone or containing 40 μM IAA-Ala. Error bars represent sos of the mean root lengths (n = 12). B, Seedlings were grown under white light on vertical plates lacking hormone at either 22°C or 28°C. Error bars are sos of the mean root lengths (n = 12). C, Seedlings were grown under either white light or yellow long-pass filters or in darkness in the presence or absence of 15 mM Suc at 22°C or 28°C. Error bars represent sos of the mean root lengths of 8-d-old seedlings (n = 12). D, For all hormones except ABA, seedlings were grown at 28°C for 8 d on the indicated concentration of each hormone. For ABA, 4-d-old seedlings were transferred to ABA and root growth 4 d after transfer was measured. Error bars represent sos of the mean root lengths (n = 12).
of recombination events in an F2 outcrossed population using PCR-based markers (Table I; Bell and Ecker, 1994), we mapped IAR4 to a 45-kb region 35 cM from the top of chromosome 1 between the markers F3I6.8 and F3I6.17 (Fig. 4A). We sequenced the coding regions of the predicted genes in this interval and identified a single base change, a C-to-T substitution in the coding region of At1g24180 (F3I6.11). A C-to-T base change is consistent with an ethylmethane sulfonate-induced mutation and converts a conserved Arg residue at position 121 to a Cys. To confirm that the mutation we identified in At1g24180 is responsible for the iar4 mutant phenotype, we transformed the mutant with a genomic construct expressing the wild-type version of the gene from its own 5' and 3' regulatory sequences (see “Materials and Methods”). As shown in Figure 4B, this construct restores IAA-Ala sensitivity and normal root elongation to iar4-1, indicating that we have identified the IAR4 gene.

To determine whether the missense mutation identified in iar4-1 was likely to confer a complete loss of function, we identified a second iar4 allele from the Salk Institute Genomic Analysis Laboratory collection (Alonso et al., 2003). The iar4-2 allele (SALK_011308) contains a T-DNA insertion in the second intron of IAR4 (Fig. 4A) and is likely to be a null allele. Like iar4-1, iar4-2 is resistant to the inhibitory effects of IAA-Ala on root elongation (Fig. 4C). Unlike the iar4-1 allele in the Wassilewskija (Ws) accession, the iar4-2 allele in the Columbia (Col-0) accession is not significantly defective in root elongation on unsupplemented medium (Fig. 4C).

As shown in Figure 5A, the predicted IAR4 protein is 81% identical to a previously characterized Arabidopsis mitochondrial PDH E1α-subunit (At1g59900; Luethy et al., 1995). Like the previously described subunit, IAR4 is predicted to have a mitochondrial targeting sequence by the iPSORT (Bannai et al., 2002) and TargetP (Emanuelsson et al., 2000) programs. In contrast, IAR4 is only 32% identical to a previously characterized Arabidopsis plastidic PDH E1α-subunit (At1g10190; Johnston et al., 1997), but the Arg residue mutated to a Cys in iar4-1 is conserved even in this distantly related E1α-subunit (Fig. 5A). These three proteins are the only apparent PDH E1α-subunits encoded in the Arabidopsis genome (The Arabidopsis Genome Initiative, 2000).

The PDH complex converts pyruvate to acetyl-CoA, thereby linking glycolysis to the Krebs cycle (Mooney et al., 2002). To test if the iar4-1 root elongation defect may be in part due to a deficiency in Krebs cycle intermediates, we examined the ability of citrate to rescue the short root phenotype of iar4-1. As shown in Figure 6B, the short root of iar4-1 is not rescued by citrate. Fumaric acid is the Krebs cycle intermediate that accumulates to the highest levels in Arabidopsis plants (Chia et al., 2000). We examined fumaric acid levels in 8-d-old iar4-1 and wild-type seedlings using gas chromatography (Chia et al., 2000) and found that iar4 and wild type accumulate similar levels of fumaric acid (Fig. 6D). These results suggest that fumaric and citric acids are not limiting in the iar4 mutant.

To test for β-oxidation defects we assessed the ability of iar4-1 to develop in the dark in the presence or absence of Suc. iar4 germinates and develops normally under these conditions (Fig. 3C), suggesting that iar4 catabolizes seed storage lipids normally and can metabolize Suc similarly to wild type. We also tested iar4-1 root elongation on a range of Suc concentrations to determine if the mutant has altered Suc sensitivity and found that iar4-1 responds to Suc

### Table I. Markers used in the positional cloning of IAR4

<table>
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<tr>
<th>Marker*</th>
<th>Enzyme</th>
<th>Size of Product (bp)</th>
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<tr>
<td></td>
<td></td>
<td>Col</td>
<td>Ws</td>
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<tr>
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<td>cut</td>
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*Markers reveal polymorphisms when cut with the indicated restriction enzymes following PCR amplification with the indicated primers, except for F3I6.23, which is a single base polymorphism that requires sequencing the PCR amplification product for detection.
similarly to wild type (Fig. 6A). In addition, we examined the response of iar4-1 to increasing concentrations of ethanol to see if the mutant would be supersensitive to ethanol because of a buildup of this pyruvate metabolite. We found that iar4-1 responds to ethanol similarly to wild type (Fig. 6C).

A Mutation in a Second Mitochondrial PDH Elα Subunit

To determine if IAA-Ala resistance can result from a general defect in pyruvate metabolism, we obtained two T-DNA insertions from the Salk Institute (La Jolla, CA) Genomic Analysis Laboratory collection in At1g59900, which encodes the previously identified mitochondrial PDH Elα-subunit (Luethy et al., 1995). Sequencing the sites of the insertions (see “Materials and Methods”) revealed that the T-DNA is inserted in the middle of intron 5 in SALK_074384 (at1g59900-1) and 37 bp upstream of the initiator ATG in SALK_047438 (at1g59900-2). Homozygous lines carrying either of these alleles displayed wild-type sensitivity to IAA-Ala and other auxins (Fig. 4C and data not shown), indicating that the iar4 mutant phenotypes are not general to all defects in PDH Elα isozymes. We also found that at1g59900-1 seedlings
Figure 5. (Legend appears on next page.)
have wild-type responses to ethanol, Suc, and citrate, and that at1g59900-1 plants lack obvious morphological abnormalities (data not shown).

**DISCUSSION**

The gene defective in the iar4 mutant encodes a protein 81% identical to a characterized Arabidopsis mitochondrial PDH E1α-subunit (Luethy et al., 1995). In plants, PDH is found both in the mitochondria and the chloroplast (Mooney et al., 2002). The E1α-subunit functions as a heterotetramer with the E1β-subunit to decarboxylate pyruvate and forms an acetaldehyde conjugate with the thiamine pyrophosphate cofactor. The acetyl group is transferred from thiamine pyrophosphate to CoA-sulphydryl group via the lipoic acid prosthetic group of the E2 subunit, resulting in the release of acetyl-CoA. The E3 subunit uses FAD to reoxidize the E2 lipoyl moieties to produce FADH₂ and then transfers the proton and electrons to NAD⁺ to form NADH (Mooney et al., 2002). To our knowledge, this is the first report of a plant mutant in any part of the PDH complex.

Phenotypic analyses of the iar4 mutant suggest that IAR4 has a function related to auxin. iar4-1 has a short root on unsupplemented medium and is less sensitive than wild type to several IAA-amino acid conjugates, to some concentrations of the synthetic auxin 2,4-D, and to the ethylene precursor ACC. In contrast, iar4 responds more similarly to wild type to exogenous IAA and to other phytohormones. Both missense (iar4-1) and insertion (iar4-2) alleles have reduced sensitivity to IAA-Ala, indicating that this phenotype results from a loss of IAR4 function.

Because iar4 was identified for its resistance to IAA-Ala, it was intriguing to learn that the defective gene in this mutant encodes an apparent PDH subunit. There are several possible explanations for the IAA-Ala resistance of the iar4 mutant. One possibility is that IAR4 is a subunit of a true mitochondrial PDH complex, and disruption of IAR4 function decreases pyruvate conversion to acetyl-CoA. In the iar4 mutant, reduced acetyl-CoA levels or accumulation of an upstream component or secondary metabolite could indirectly affect auxin metabolism by depleting precursors or inhibiting required reactions (Fig. 7A). However, citrate, which enters the TCA cycle downstream of acetyl-CoA, fails to rescue the iar4 mutant root elongation defect; the mutant accumulates normal levels of fumaric acid; and ethanol, which is derived from pyruvate, does not exacerbate the mutant phenotype (Fig. 6). The iar4 mutant also responds like wild type to Suc (Fig. 6). We expected that Suc might either rescue the root elongation defect due to increased flux through glycolytic pathways or result in increased sensitivity due to a buildup of pyruvate or side products. We did not observe either of these effects. Although these are negative results, none provide evidence that iar4 is defective in processes that might be expected to be affected by mitochondrial PDH.

We also examined insertional mutants in At1g59900, the other Arabidopsis mitochondrial PDH E1α-subunit gene. Homozygous plants carrying an insertion before exon 6 of At1g59900, which encodes a mitochondrial E1α protein 81% identical to IAR4 (Luethy et al., 1995), are viable and have wild-type responses to IAA-Ala. Because iar4 is resistant to IAA-Ala, whereas a mutant defective in the other mitochondrial PDH E1α responds normally to IAA-Ala, it is possible that IAR4 acts in IAA-Ala metabolism or response while the other E1α-subunit acts in mitochondrial pyruvate metabolism. Some functional redundancy between IAR4 and At1g59900 in pyruvate metabolism is also likely, as both iar4 and at1g59900-1 lack dramatic growth defects and have wild-type responses to ethanol, Suc, and citrate. An essential role for PDH E1α activity is suggested by the recent report of male sterility resulting from antisense expression of a sugar beet PDH E1α gene in tobacco anther tapetum (Yui et al., 2003). It is likely that a double mutant between the iar4 and at1g59900-1 mutants described here would reveal any functional redundancy between these two isozymes.

Interestingly, in addition to the two predicted mitochondrial E1α-subunits (IAR4/At1g24180 and At1g59900), there are three predicted E1β-subunits (At1g30120, At2g34590, and At5g50850) in Arabidopsis. Although only a single combination (At1g59900

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**Figure 5.** IAR4 is similar to mitochondrial PDH E1α-subunits. A, Sequences from predicted PDH E1α-subunits were aligned with the MegAlign program (DNASTAR, Inc., Madison, WI) using the Clustal W method. Amino acid residues identical in at least four of the sequences are boxed in black. The position of the Arg residue mutated to a Cys in iar4-1 is highlighted with a gray box and a triangle indicates the position of the intron interrupted by a T-DNA in iar4-2. The protein predicted from the IAR4 cDNA is compared to predicted mitochondrial E1α sequences from Arabidopsis (At1g59900; Luethy et al., 1995); sugar beet (By; Yui et al., 2003), potato (St; Grol et al., 1995), maize (Zm; Thelen et al., 1999), budding yeast (Sc; Behal et al., 1989), and human (Hs; GenBank accession no. AAH30697) and the plastid PDH E1α from Arabidopsis (At1g01090; Johnston et al., 1997). Also included are sequences from rice (Os) and cotton (Gh) plant genome projects assembled in the TIGR Gene Index Database (URL: http://www.tigr.org/db/). B, Phylogenetic tree of IAR4 and its relatives. The tree reconstructs the evolutionary relationship between selected characterized and putative PDH E1α-subunits. The proteins were aligned as described in panel A, and the unrooted phylogram was generated by using PAUP 4.05b (Swofford, 2001). The bootstrap method was performed for 200 replicates with a distance optimality criterion, and all characters were weighted equally. Along with the proteins from panel A, additional sequences compared in panel B are from rat (Rr; Matuda et al., 1991), Sinorhizobium meliloti (Sm; GenBank accession no. NP_385531), and TIGR Gene Index Database sequences from Medicago truncatula (Mt), wheat (Ta), tomato (Le), and rice (Os). Shaded ovals highlight the plant proteins predicted to be mitochondrial (left) or plastidic (right).
E1α with At5g50850 E1β) has been heterologously expressed, purified, and tested for activity in vitro (Szurmak et al., 2003), a variety of E1a2b2 heterotetramers may exist in vivo, and these different enzymes may display different catalytic or regulatory properties. Rather than or in addition to acting on pyruvate, IAR4 may function with other PDH subunits to catalyze the conversion of indole-3-pyruvate (IPA) to indole-3-acetyl-CoA (IAA-CoA; Fig. 7B). In several plant-associated microbes, IPA is an intermediate in an IAA biosynthetic pathway in which a Trp aminotransferase converts Trp to IPA, and an IPA decarboxylase converts IPA to indole-3-acetaldehyde, which is then oxidized to IAA (Koga, 1995). IPA is present in Arabidopsis (Tam and Normanly, 1998), but the pathways by which it is formed and the products to which it contributes are unknown (Bartel et al., 2001). If IAR4 functions as a subunit of an IPA dehydrogenase that converts IPA to IAA-CoA, this IAA-CoA could then be hydrolyzed to release IAA or could provide activated IAA as a precursor to amide-linked IAA conjugates such as IAA-amino acids (Fig. 7B). In this case, certain iar4 mutant tissues could have reduced levels of IAA and/or IAA-Ala, which might lead to the observed IAA-Ala resistance. The stimulation by CoA of in vitro IAA ester formation led to the initial suggestion that IAA-CoA might be an intermediate in IAA conjugate formation (Kopcewicz et al., 1974). Biochemical studies have shown that formation of IAA-Asp, which is thought to function in auxin detoxification (Normanly, 1997), has both auxin-inducible and constitutive components (Venis, 1972). It is likely that a recently described family of IAA adenylating enzymes act in at least some pathways of IAA conjugate formation (Staswick et al., 2002), but it is possible that distinct enzymes and pathways are used to synthesize conjugates used for storage, constitutive detoxification, and inducible detoxification of IAA.

To explore the likelihood that Arabidopsis encodes mitochondrial PDH E1α-subunits with different roles, we undertook a phylogenetic analysis of available plant sequences. Examination of assembled cDNAs from various plant genome projects (TIGR Gene Index Databases; URL: http://www.tigr.org/tdb/tgi; Quackenbush et al., 2001) revealed that the closest sequenced IAR4 homolog is TC17531 from cotton, which is 82% to 83% identical to both IAR4 and At1g59900 (Fig. 5A). Phylogenetic analysis does not reveal whether this cotton sequence is more closely related to IAR4 or At1g59900 (Fig. 5B). When additional plant genome sequences are complete, it will be interesting to learn whether other plants, like...
Arabidopsis and Medicago, encode multiple apparent mitochondrial Elα-subunits (Fig. 5B).

Our understanding of IAA homeostasis is far from complete, but further characterization of IAR4 may reveal the importance of IAA to IAA-CoA conversion in IAA or conjugate biosynthesis or may provide a link between more general glycolytic pathways and IAA biosynthesis and metabolism.

MATERIALS AND METHODS

Plant Growth Conditions

Arabidopsis accessions Wassilewskija (Ws) and Columbia (Col-0) were used as wild type for iar4-1 and iar4-2, respectively. For determination of root length, seeds were surface sterilized (Last and Fink, 1988) and sown on plant nutrient medium (PN; Haughn and Somerville, 1986) solidified with 0.6% (w/v) agar and supplemented with 15 mM Suc (PNS) unless otherwise noted. Plates were sealed with gas-permeable surgical tape (LeTeic, Minnetonka, MN). For growth in soil, plants were either transferred from PNS or sown directly in soil (Metromix 200; Scotts, Marysville, OH) and grown at 22°C under continuous illumination with cool-white fluorescent bulbs (approximately 200 µm s⁻¹; Sylvania, Versailles, KY).

Unless otherwise indicated, plates were incubated at 22 or 28°C under yellow long-pass filters (25-45 µm s⁻¹) to slow the breakdown of indolic compounds (Stasinopoulos and Hangarter, 1990). IAA-α-amino acid conjugates were from Aldrich (Milwaukee, WI) or were synthesized as described (LeClere et al., 2002). Conjugates were diluted to 22 or 100 mM stocks in 50% (v/v) ethanol. Remaining hormone stocks were diluted from stocks in 100% ethanol. For testing root elongation onABA, 4-d-old seedlings were transferred from PNS to plates containing ABA, the positions of the root tips were marked, plates were incubated vertically for another 4 d, and root growth following transfer was measured. For dark grown seedlings, plates were incubated for 1 d under white light (120 µm s⁻¹) to induce germination then wrapped in aluminum foil for the indicated number of days.

Mutant Isolation

The iar4-1 mutant was isolated as described previously for iar4-1 (Davies et al., 1999) from pools of Ws mutagenized with ethylmethane sulfonate. The iar4-1 mutant was backcrossed to the parental Ws line five times to remove extraneous mutations prior to phenotypic analysis. The iar4-2 mutant is a sequence-indexed Arabidopsis T-DNA insertion mutant (SALK_011308) isolated by the Salk Institute Genomic Analysis Laboratory (Alonso et al., 2003) from individuals with the longest roots and screened with PCR-based polymorphic markers (Konieczny and Ausubel, 1993; Bell and Ecker, 1994), including the newly developed markers shown in Table I. The predicted open reading frames in the mapping interval were amplified by PCR from genomic DNA prepared from the iar4-1 mutant. For IAR4, two pairs of oligonucleotides were used for amplification (F3I6.11-2: TTATACATGTT-GACCTGACTTCCAACCC plus F3I6.11-7: TGAAAACATCTCCTTGGG-GAAGCAAG and F3I6.11-3: ATGTCAGGCTTTGATATTGTTGAGAACAG and F3I6.11-6: GGTTTGACCTTTCCATAAAACGGGCTTCCG) with a program of 40 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 2 min. Amplification products were gel purified and sequenced directly using an automated DNA sequencer (Lone Star Labs, Houston) and the primers used for amplification plus additional primers (F3I6.11-4: TAGCTGGAATG-GAACGCTACATGGGAG and F3I6.11-5: AGGAACACTGCTCACAAGT-CAGG).

To confirm the splicing pattern of the predicted IAR4 gene, an EST clone corresponding to a full-length IAR4 cDNA (213P1217) was obtained from ABRC. The insert of this cDNA was sequenced using vector-derived and internal primers. The GenBank accession number for the IAR4 cDNA isAY135561.

Generation of Rescue Constructs and Transgenic Plants

To generate a genomic IAR4 rescue construct, a 3.4-kb SpeI fragment was isolated from the bacterial artificial chromosome F3I6 (GenBank accession no. AC002396). This fragment was subcloned into the Smal site of the plant transformation vector pbENEBial, a plasmid offering blue/white selection and kanamycin and ampicillin selection in bacteria, BASTA selection in soil, and plasmid rescue capabilities in transgenic plants (LeClere, 2002). The resultant plasmid (pbENEB-IAR4) was electroporated into Agrobacterium strain GV3101 (Koncz et al., 1992) and the floral dip method (Clough and Bent, 1998) was used to transform homozygous iar4-1 mutant plants that had been backcrossed to Ws five times. T0 plants were selected in soil as previously described (LeClere and Bartel, 2001). Lines homozygous for the transgene were selected by examining the BASTA resistance of T1 seedlings.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requester.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numberAY135561.

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One hundred 8-d-old seedlings grown under white light on filter paper overlaid on PNS were harvested and stored at −80°C until analysis. Fumarate content was assayed using gas chromatography as previously described (Chia et al., 2000). Fumarate levels were normalized by dividing the area of the fumarate peak by the area of the palmitoleic acid (16:1) peak and are expressed in arbitrary units.
LITERATURE CITED


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