IAR3 Encodes an Auxin Conjugate Hydrolase from Arabidopsis

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Amide-linked conjugates of indole-3-acetic acid (IAA) are putative storage or inactivation forms of the growth hormone auxin. Here, we describe the Arabidopsis iar3 mutant that displays reduced sensitivity to IAA–Ala. IAR3 is a member of a family of Arabidopsis genes related to the previously isolated ILR1 gene, which encodes an IAA-amino acid hydrolase selective for IAA–Leu and IAA–Phe. IAR3 and the very similar ILL5 gene are closely linked on chromosome 1 and comprise a subfamily of the six Arabidopsis IAA-conjugate hydrolases. The purified IAR3 enzyme hydrolyzes IAA-Ala in vitro. iar3 ilr1 double mutants are more resistant than either single mutant to IAA-amino acid conjugates, and plants overexpressing IAR3 or ILR1 are more sensitive than is the wild type to certain IAA-amino acid conjugates, reflecting the overlapping substrate specificities of the corresponding enzymes. The IAR3 gene is expressed most strongly in roots, stems, and flowers, suggesting roles for IAA-conjugate hydrolysis in those tissues.

INTRODUCTION

Indole-3-acetic acid (IAA) is involved in virtually all aspects of plant growth and development (Davies, 1995). Plants produce active IAA de novo synthesis and by hydrolyzing IAA conjugates (reviewed in Normanly et al., 1995; Bartel, 1997; Normanly, 1997). IAA conjugation activity is widely distributed in the plant kingdom from mosses to angiosperms (Sztein et al., 1995), and most IAA in plant tissues is conjugated via its carboxyl group to sugars, high molecular weight glycans, amino acids, or peptides (Cohen and Bandurski, 1982; Bandurski et al., 1995). Different conjugates may perform different functions in the plant. For example, IAA–Asp is an intermediate in IAA destruction (Tsurumi and Wada, 1986; Monteiro et al., 1988; Tuominen et al., 1994; Östin et al., 1998). Other conjugates may serve as reservoirs of inactive IAA that can be hydrolyzed to supply the plant with active hormone, as in maize germination when conjugate hydrolysis provides free IAA to the developing seedling (Epstein et al., 1980).

IAA-conjugate hydrolases release free IAA from the conjugate form and thus are likely to play an important role in regulating free IAA levels. These hydrolases have been detected in bacteria (Chou et al., 1996) and in a variety of plants (Hall and Bandurski, 1986; Cohen et al., 1988; Kowalczyk and Bandurski, 1990; Jakubowska et al., 1993; Kuleck and Cohen, 1993; Bartel and Fink, 1995; Ludwig-Müller et al., 1996). The auxin effects of IAA-amino acid conjugates correlate with their hydrolysis rates both in tissue culture (Hangarter and Good, 1981) and in bean internode curvature assays (Bialek et al., 1983). IAA–Ala hydrolases have been partially purified from bean (Cohen et al., 1988) and carrot (Kuleck and Cohen, 1993). Extracts of Chinese cabbage hydrolyze IAA–Ala, IAA–Asp, and IAA–Phe but not IAA conjugated to inositol or the amino acids Gly, Val, or Ile (Ludwig-Müller et al., 1996). Interestingly, infection with Plasmodiophora brassicae (which causes clubroot disease) correlates with a dramatic increase in the rate of IAA–Asp hydrolysis (Ludwig-Müller et al., 1996). This induction of one specific hydrolytic activity in response to a particular challenge suggests that various conjugate hydrolases might supply free IAA in response to a variety of needs.

We are exploring the role of IAA-conjugate hydrolysis in plant growth and development by using the model dicot Arabidopsis. Several exogenous IAA conjugates mimic IAA (Feung et al., 1977; Hangarter et al., 1980; Bialek et al., 1983; Magnus et al., 1992a, 1992b; Bartel and Fink, 1995; Soskic et al., 1995), suggesting either that these conjugates are auxins or that they are hydrolyzed to release IAA. We isolated a gene encoding an IAA-conjugate hydrolase by exploiting this observation. The Arabidopsis ilr1 (for IAA–Leu-resistant) mutant is able to elongate roots on inhibitory concentrations of IAA–Leu (Bartel and Fink, 1995). Two mutants resistant to IAA–Phe, icr1 (for IAA–conjugate resistance) and icr2, were isolated using a similar strategy (Campanella et al., 1996). The gene defective in the ilr1 mutant was cloned using a map-based approach and shown to encode an IAA-amino acid hydrolase selective for IAA–Leu and IAA–Phe.

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among the conjugates tested. ILR1 is a member of a gene family in Arabidopsis (Bartel and Fink, 1995). Additional IAA-amino acid conjugate—insensitive mutants may have defects in other ILR1 gene family members. Here, we report the isolation of the iar3 (for IAA–Ala—resistant) mutant and demonstrate that it is mutated in an ILR1 homolog that encodes an IAA-Ala—specific hydrolase. We also report the isolation of two additional ILR1-like genes, ILL3 and ILL5, bringing the total number of known IAA-conjugate hydrolase–related genes in Arabidopsis to six.

RESULTS

Isolation and Characterization of iar3 Mutants

The effects of certain IAA-amino acid conjugates mimic those of IAA when included in the growth media. One phenotype caused by these compounds is an inhibition of root elongation. This phenotype has been used to isolate mutants with decreased sensitivity to several conjugates (Campanella et al., 1996), including the ilr1 mutant (Bartel and Fink, 1995). To isolate mutants in other members of the ILR1 gene family, we screened for additional IAA–amino acid—insensitive mutants.

We isolated four recessive alleles of iar3 from three different screens for increased root elongation on inhibitory concentrations of IAA conjugates (see Methods). iar3-1 was isolated from the progeny of ethyl methanesulfonate (EMS)—mutagenized wild-type (ecotype Wassilewskija [WS]) seeds screened on 50 μM IAA–Ala. We also screened for ilr1-1 enhancers to uncover hydrolases with overlapping substrate specificity with ILR1. The ilr1-1 mutant originally was isolated due to its increased root elongation on 50 μM IAA–Leu (Bartel and Fink, 1995). Three iar3 alleles were isolated by screening the progeny of EMS-mutagenized ilr1-1 seeds on 100 μM IAA–Leu (iar3-2 and iar3-4) or 70 μM IAA–Phe (iar3-3). The iar3 mutant alleles were backcrossed to the WS ecotype to remove unlinked mutations (including the ilr1-1 mutation) and outcrossed to the Columbia (Col-0) ecotype for mapping (see Methods). Using polymerase chain reaction (PCR)—based polymorphic markers (Konieczny and Ausubel, 1993; Bell and Ecker, 1994), we mapped each of the iar3 mutant alleles to the lower arm of chromosome 1 between nga280 (Bell and Ecker, 1994) and GAPB (Konieczny and Ausubel, 1993), ~8 centimorgans from nga280.

To compare the responses of the mutant alleles with different IAA-amino acid conjugates, we germinated the iar3 mutants, the ilr1-1 mutant, and iar3 ilr1-1 double mutants on plates containing various IAA–amino acid conjugates and measured root elongation after 8 days. The results of this analysis are shown in Figure 1. In contrast to the ilr1-1 mutant, which is resistant to IAA–Leu, moderately resistant to IAA–Phe, and not resistant to IAA–Ala, the iar3 mutants are resistant to IAA–Ala but are not resistant to IAA–Leu or IAA–Phe. The iar3 ilr1-1 double mutants are more resistant to all three conjugates, particularly IAA–Phe. The strongest iar3

![Figure 1.](image-url)
allele, iar3-2, also is slightly resistant to free IAA, especially in combination with the ilr1-1 mutant (see Discussion).

Isolation of ILR1-like Genes

Because the iar3 mutant is insensitive to IAA conjugates, we explored the possibility that IAR3 might encode an IAA-conjugate hydrolase by isolating ILR1-like genes and comparing their map positions with that of the mutant. We amplified Arabidopsis genomic DNA by using degenerate PCR (see Methods) with oligonucleotides designed from conserved regions among ILR1 and two ILR1-like genes, ILL1 and ILL2 (Bartel and Fink, 1995). As shown in Figure 2, this PCR resulted in five discrete bands (reflecting different intron sizes) when separated by electrophoresis on an agarose gel. Subcloning, restriction mapping, and sequencing of these products revealed that they are derived from six different genes. In addition to the previously isolated ILR1, ILL1, and ILL2 genes, this analysis revealed three new genes, ILL3, ILL5, and the IAR3 gene that was disrupted in the iar3 mutant (see below). We also found partial cDNA clones of IAR3 and ILL3 by searching the Arabidopsis expressed sequence tag (EST) database (Newman et al., 1994) for ILR1-like genes.

Genomic clones representing these three new genes and ILL1 and ILL2 were isolated by hybridizing a cosmids library with radioactive probes (Olszewski et al., 1988), and the coding regions were sequenced (see Methods). The coding regions of the ILL1 and ILL2 genes were found in a head-to-tail arrangement 790 bp apart on a single cosmids, and IAR3 and ILL5 were similarly found on a single cosmids, although the exact distance between them remains undetermined. Full-length IAR3 and ILL3 cDNA clones were isolated by hybridization from a plasmid library (Minet et al., 1992) and sequenced. The predicted amino acid sequences of the ILR1-like proteins are between 44 and 87% identical to one another and are aligned in Figure 3.

The presence in a library of cDNAs for ILR1, ILL1, ILL2 (Bartel and Fink, 1995), ILL3, and IAR3 indicates that these genes are all expressed. We have not isolated an ILL5 cDNA, and the EST database currently lacks ILL5 cDNAs. Therefore, in the protein alignment (Figure 3), we conceptually spliced the ILL5 gene as the IAR3, ILL1, ILL2, ILL3, and ILR1 genes are spliced. However, the ILL5 3′ splice acceptor site for intron 2 is changed from the canonical AG:G (Brown et al., 1996) found in the other conjugate hydrolase genes to AT:G. We confirmed that this reflected genomic sequence and not a mutation in the library by sequencing the PCR-amplified splice junction from Col-0 genomic DNA (see Methods). Thus, the ILL5 gene, if expressed, may not be spliced to give a functional protein. However, similar analysis of genomic DNA from the Landsberg erecta (Ler) and WS ecotypes indicated that these strains have the typical AG:G putative splice acceptor sites. Thus, functional ILL5 protein may be restricted to certain Arabidopsis ecotypes. In preliminary experiments, we have not detected the

Figure 2. Structure of IAR3 and Related Genes Isolated By Using Degenerate PCR.

The genomic structure of the Arabidopsis IAA-conjugate hydrolase genes is shown at left. Exons are shown as rectangles, and introns are shown as thick lines. Arrows above exons 1 and 3 indicate the positions of degenerate PCR primers designed from regions of similarity in ILR1, ILL1, and ILL2 protein sequences (open rectangles) that were used to amplify genomic DNA. At right is a 2% agarose gel stained with ethidium bromide. Lane 1 contains the products of PCR amplification by using genomic DNA from the ecotype WS as a template, and lane 2 contains molecular length markers, given in kilobases at right.

ILL5 message by reverse transcriptase–PCR (RT-PCR) in RNA prepared from various tissues of Col-0 wild-type plants (J. Lasswell, R.T. Davies, and B. Bartel, unpublished data).

IAR3 Is an ILR1 Homolog

We developed PCR-based polymorphic markers to map the ILR1-like genes (see Methods). As summarized in Figure 4, we mapped ILL1 and ILL2 between markers m435 (4.6% recombination) and g2368 (9.3% recombination) on the bottom of chromosome 5. ILL3 also mapped to the bottom of chromosome 5 and was tightly linked to m435 (zero recombinants in 64 chromosomes scored). Recently, these three genes also were sequenced by the Kazusa DNA Research Institute (http://www.kazusa.or.jp/arabi/chr5/map/20-22Mb.html) as part of P1 clone MK19, which includes the ILL1 and ILL2 genes, and TAC clone K18G13, which contains the 3′ end of the ILL3 gene. The reported physical positions of these genomic clones agree with our genetic mapping data.

One of the new genes mapped to a position on chromosome 1 indistinguishable from that of the iar3 mutant. Therefore, we PCR-amplified this gene from genomic DNA prepared from each of the four iar3 mutant alleles and the parental strain (WS) and sequenced these PCR products directly (Ausubel et al., 1995). Each allele had a single nucleotide transition consistent with EMS mutagenesis that altered an amino acid conserved in Arabidopsis hydrolases. These changes are shown in Table 1 and Figure 3. These sequencing data indicate that we have identified the IAR3
The similarity of the IAR3 protein to the ILR1 amidohydrolase and the IAA–Ala resistance of the iar3 mutant strongly suggested that IAR3 is an IAA–amino acid hydrolase. To test this conclusion directly, we expressed the IAR3 cDNA in *Escherichia coli* as a glutathione S-transferase (GST) fusion, purified the fusion protein on glutathione–agarose, and tested the resultant protein for conjugate hydrolysis (see Methods). We also generated the iar3-1 mutant cDNA by changing serine 106 to leucine (Table 1 and Figure 3) and made the corresponding GST fusion protein. We expressed and purified GST from the empty vector and tested its conjugate hydrolysis activity as a control. SDS-PAGE analysis of the purified proteins is shown in Figure 5. The pH optimum for IAR3 in Tris–HCl buffer is pH 8.0 (see Methods). As shown in Table 2, the GSTIAR3 protein (see Methods) hydrolyzed IAA–Ala and IAA–Gly efficiently and IAA–Phe, IAA–Leu, and IAA–Val much less efficiently. The GSTIAR3 protein did not detectably hydrolyze IAA–Ile. The GSTiar3-1 fusion protein still hydrolyzed IAA–Ala and IAA–Gly but at 3% of the rate of the GSTIAR3 protein, indicating that the iar3-1 mutation severely impairs the encoded hydrolase. IAA–Phe, IAA–Leu, IAA–Val, and IAA–Ile were not detectably hydrolyzed by the mutant GSTiar3-1 protein. GST alone did not hydrolyze any of the conjugates tested.

**IAR3**

Expression

To determine which tissues normally express IAR3, we analyzed RNA gel blots. Figures 6A and 6B show that the IAR3
gene is expressed most strongly in roots, stems, and inflorescences and is less abundant, but detectable, in other tissues. The IAR3 probe does not cross-hybridize with the ILR1, ILL1, ILL2, or ILL3 genes under the conditions used (data not shown). Although ILL5, the closest homolog of IAR3, does cross-hybridize with IAR3 (J. Lasswell and B. Bartel, unpublished data), we do not see a larger transcript consistent with ILL5 expression, and we have not detected ILL5 message by RT-PCR in the same RNA samples (J. Lasswell, R.T. Davies, and B. Bartel, unpublished data). Thus, the signal in Figure 6A is likely to be specific for the IAR3 message.

To determine the consequences of IAA-conjugate hydrolase overexpression, we constructed transgenic plants carrying the IAR3 or ILR1 cDNAs driven by the cauliflower mosaic virus 35S promoter (see Methods). Roots of homozygous lines expressing the transgenes were measured following growth on various IAA conjugates. We used a concentration of conjugates (10 or 20 μM) in these experiments that moderately inhibited wild-type root growth (28 to 40%) to facilitate the detection of increases in sensitivity. As shown in Figure 7, overexpression of IAR3 increased sensitivity to IAA–Ala but did not significantly alter sensitivity to IAA–Phe or IAA–Leu. By contrast, overexpression of ILR1 increased sensitivity to IAA–Phe and IAA–Leu but did not significantly alter sensitivity to IAA–Ala (Figure 7). Thus, the sensitivity to exogenous conjugates in vivo mirrors the differences in substrate specificity between these two enzymes detected in vitro.

### DISCUSSION

#### A Family of IAA-Conjugate Hydrolases

We have isolated a family of Arabidopsis genes that resemble the IAA-conjugate hydrolase–encoding ILR1 gene (Bartel and Fink, 1995). Each of the six genes has five exons interrupted by introns in corresponding positions (Figures 2 and 3). The predicted gene products are between 44 and 87% identical to one another, and the two pairs of genes that most resemble one another are closely linked in the genome (Figure 4). ILL1 and ILL2 (87% identical at the amino acid level) are arranged in a direct repeat on chromosome 5, and IAR3 and ILL5 (83% identical at the amino acid level) are within 20 kb of one another on chromosome 1. These gene pairs presumably result from relatively recent genomic duplication events. After ILL5, the IAR3 enzyme is related more closely to ILL2 and ILL1 (59 and 58% identical, respectively) than to ILR1 (47%) or ILL3 (45%).

Each of the six Arabidopsis conjugate hydrolase–like genes potentially encodes an ~48-kD protein. Analysis of the predicted protein products suggests that several of these hydrolases reside in the endoplasmic reticulum (ER) lumen. Each protein has a single stretch of hydrophobic amino acids near the N terminus that is an apparent cleavable signal sequence for ER targeting (Nakai and Kanehisa, 1992), and the IAR3, ILL5, and ILL1 proteins terminate in the sequence (H or K)DEL (Figure 3), which signals retrieval of plant proteins to the ER lumen (Bednarek and Raikhel, 1992). ILR1 (KSEL) and ILL2 (HEEL) end in sequences very similar to this consensus sequence and might also localize to the ER. In contrast, ILL3 terminates in a completely different sequence (KSGD) that is unlikely to signal ER retention. Thus, primary sequence analysis suggests that the conjugate hydrolases might be directed to multiple subcellular compartments.

The ILR1 and IAR3 enzymes have distinct substrate preferences in vitro and in vivo. ILR1 hydrolyzes IAA–Phe and IAA–Leu six to eight times faster than it does IAA–Ala, IAA–Gly, or IAA–Val (Bartel and Fink, 1995), whereas IAR3 hydrolyzes IAA–Ala more than six times faster than IAA–Gly and 200 times faster than conjugates with Val, Phe, or Leu (Table 2). IAA–Ile is not detectably hydrolyzed by either enzyme. These differences also are seen after overexpression of the hydrolases in transgenic plants (Figure 7). This heterogeneity in substrate specificity may imply that Arabidopsis has a
variety of IAA–amino acid conjugates. However, determination of the in vivo relevance of these differences awaits the identification of the endogenous Arabidopsis auxin conjugates. There are already several reports of IAA–Ala in other plants. IAA–Ala has been found with gas chromatography–mass spectrometry in Norway spruce needles (Östin et al., 1992). In addition, IAA-Ala is reported among the conjugates formed after application of IAA to crown gall tissue (Feung et al., 1976), and IAA–Ala cochromatographs by using thin-layer chromatography with a conjugate formed after exogenous IAA application to a variety of uninfected plants, including horsetail, terrestrial ferns, and the gymnosperm Pinus thunbergiana (Sztein et al., 1995).

IAR3-like activity has been observed in a wide variety of plants. IAA–Ala hydrolases have been detected in extracts of Chinese cabbage (Ludwig-Müller et al., 1996) and partially purified from bean (Cohen et al., 1988) and carrot (Kuleck and Cohen, 1993). IAA–Ala is among the most active IAA–amino acid conjugates in bioassays not only in Arabidopsis (Figure 1; Bartel and Fink, 1995; Campanella et al., 1996) but also in oat (Feung et al., 1977), bean (Bialek et al., 1983), pea (Hangarter et al., 1980; Hangarter and Good, 1981), black nightshade, and tomato (Magnus et al., 1992b).

Comparison of the Arabidopsis IAA-conjugate hydrolase genes with sequences from other plants in the GenBank database reveals that there are members of this hydrolase family in rice, cotton, apple, and flax (data not shown). IAR3 is related more closely (81% identical over 106 amino acids) to one of these rice sequences (GenBank accession number RICC1732A) than to ILR1 (49% identical over the same region), suggesting that IAR3 and ILR1 diverged from one another well before monocots and dicots separated. Thus, there appears to have been selection for diverse hydrolase activities early in angiosperm evolution.

Interestingly, an Arabidopsis cDNA named JR3, which is 96% identical to IAR3 at the nucleotide level (3-bp substitutions and 12-bp insertions), was isolated as a jasmonic acid (JA)-induced gene by using differential display (Titarenko et al., 1997). JR3 also is induced rapidly and transiently by wounding, both locally in wounded tissue and systemically in unwounded leaves of injured plants (Titarenko et al., 1997; Rojo et al., 1998). Our demonstration that IAR3 functions to release free IAA from conjugates supports the hypothesis that JR3 might be involved in feedback inhibition of the JA response (Rojo et al., 1998). It will be interesting to

Table 1. Mutations Found in iar3 Alleles

<table>
<thead>
<tr>
<th>Allele</th>
<th>Mutagen</th>
<th>Codon Change</th>
<th>Amino Acid Change</th>
<th>Restriction Enzyme Site Change</th>
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<tr>
<td>iar3-1</td>
<td>EMS</td>
<td>TCG→TGG</td>
<td>Ser-106→Leu</td>
<td>Destroys XhoI</td>
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<tr>
<td>iar3-2</td>
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<td>Gly-124→Glu</td>
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<tr>
<td>iar3-3</td>
<td>EMS</td>
<td>GCT→ACT</td>
<td>Ala-130→Thr</td>
<td>Destroys Alul</td>
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<tr>
<td>iar3-4</td>
<td>EMS</td>
<td>GGA→GAA</td>
<td>Gly-126→Glu</td>
<td>Destroys MNil</td>
</tr>
</tbody>
</table>

aNucleotide changes are underlined.
determine whether the other conjugate hydrolase genes are regulated similarly and whether the iar3 or iar3 ilr1 mutants show altered responses to JA, wounding, or microbial pathogens.

Mutants with Defective IAA-Conjugate Hydrolases

We have shown that two IAA-amino acid—insensitive mutants, ilr1 (Bartel and Fink, 1995) and iar3, are defective in specific IAA-conjugate hydrolases; decreased IAA-conjugate cleavage probably causes these IAA-amino acid insensitive phenotypes. The additive interaction of the ilr1 and iar3 mutations (Figure 1) parallels the overlapping substrate specificities of the corresponding enzymes in vitro (Table 2; Bartel and Fink, 1995). The IAA-Ala—resistant phenotype of the iar3 mutant is observed in a root elongation assay (Figure 1), which is consistent with the IAR3 mRNA being expressed in root tissue (Figure 6). This expression suggests a role for auxin conjugate hydrolysis in root growth or development. IAR3 also is highly expressed in stems and flowers, suggesting roles for conjugate hydrolysis in these tissues as well.

The iar3 mutant is most resistant to IAA-Ala among conjugates tested and also enhances the ilr1 mutant phenotype on either IAA-Leu or IAA-Phe (Figure 1). Three of the iar3 alleles were originally isolated as ilr1 enhancers based on this phenotype. IAR3 cDNA expression from the strong viral 35S promoter caused the expected increased IAA-Ala sensitivity, whereas sensitivity to IAA-Phe and IAA-Leu was not significantly enhanced (Figure 7). The E. coli–expressed IAR3 protein efficiently hydrolyzed IAA-Ala and hydrolyzed IAA-Leu and IAA-Phe much less efficiently (Table 2). Thus, the in vitro substrate specificity of the IAR3 protein mirrors the in vivo activity of the IAR3 gene.

The four mutations in iar3 are clustered in the middle of the third exon (Figure 3). The iar3-2 and iar3-4 mutations change Gly residues that are conserved in the six Arabidopsis Auxin-Conjugate Hydrolase Mutant 371

sis enzymes and in related microbial enzymes (Bartel and Fink, 1995) to Glu residues, and the iar3-2 mutant is the strongest allele that we have isolated (Figure 1). The other two mutant alleles encode less dramatic changes. The mutation found in iar3-1 changes Ser-106 to Leu, which is the amino acid found in the analogous position in the similar hippuricase enzyme from Campylobacter jejuni (Hani and Chan, 1995). Therefore, it is unlikely that this mutation affects the overall conformation of the protein. The recombinant GSTiar3-1 protein expression and purification yields mirrored those of the wild-type fusion protein (Figure 5), but it retained <3% of the wild-type hydrolytic activity (Table 2), confirming that protein encoded by the iar3-1 allele is catalytically compromised.

iar3-2, the strongest iar3 allele, is insensitive not only to IAA-Ala but also to the inhibitory effects of IAA itself on root elongation. This small but statistically significant difference is enhanced in the iar3-2 ilr1-1 double mutant (Figure 1). This resistance may reflect a decreased rate of endogenous conjugate hydrolysis in the double mutant that allows exogenous IAA to be more efficiently inactivated. Exogenous IAA supplied to Arabidopsis is both oxidized directly and conjugated to Asp and Glu (Östin et al., 1998). IAA-Asp is metabolized further to oxidized forms (Östin et al., 1998), indicating that IAA-Asp formation in Arabidopsis is an initial step in the irreversible inactivation of exogenously supplied IAA, as has been shown in a variety of other plants (Tsurumi and Wada, 1986; Monteiro et al., 1988; Tuominen et al., 1994). Preliminary experiments show that neither ILR1 nor IAR3 hydrolyze IAA-Asp in vitro (R.T. Davies and B. Bartel, unpublished data), which is consistent with a role for this conjugate in IAA inactivation. The observation that the iar3-2 ilr1-1 double mutant is more resistant than the wild type to exogenously supplied IAA suggests that the ILR1 and IAR3 enzymes are involved in IAA homeostasis in vivo.

None of the four iar3 mutant alleles, either alone or in combination with the ilr1-1 mutation, has a dramatic morphological phenotype in the absence of IAA conjugates.

| Table 2. Hydrolysis of IAA Conjugates by IAR3 and iar3-1 |
|---------------------------------|---------------------------------|
| Substrate          | GSTIAR3 Activity |
| (IAA released; pmol min⁻¹ mg⁻¹ protein) | % of Maximum Activity | GSTiar3-1 Activity |
| (IAA released; pmol min⁻¹ mg⁻¹ protein) | % of GSTIAR3 Activity |
| IAA-Ala            | 640 ± 110           | 100                             | 4.6 ± 0.6          | 0.72 |
| IAA-Gly            | 101 ± 16            | 16                              | 2.4 ± 0.2          | 2.4  |
| IAA-Val            | 2.6 ± 1.0           | 0.41                            | <0.05              | <1.9 |
| IAA-Phe            | 1.9 ± 0.1           | 0.30                            | <0.05              | <2.6 |
| IAA-Leu            | 1.8 ± 0.3           | 0.28                            | <0.05              | <2.8 |
| IAA-Ile            | <0.05               | <0.008                          | <0.05              | —    |

*aRates shown are the mean ±SD for three time points, except for GSTIAR3 with IAA-Phe and GSTiar3-1 with IAA-Ala or IAA-Gly, which are the mean plus or minus average deviations from the mean for two time points (see Methods). Purified GST did not hydrolyze any substrate tested (<0.05 pmol min⁻¹ mg⁻¹).
Each allele has a missense mutation (Table 1), and the enzyme encoded by iar3-1 retains slight activity (Table 2). The conjugate hydrolases encoded by the ILL1, ILL2, ILL3, or ILL5 genes may provide redundancy for any essential IAR3 or ILR1 functions in vivo. The feasibility of obtaining recessive mutations in the IAR3 and ILR1 genes by using a functional assay (root elongation on IAA conjugates) does imply that the remaining hydrolases in the iar3 and ilr1 mutants do not fully compensate for the absence of IAR3 and ILR1, at least in seedlings. Ongoing efforts to obtain mutants defective in the remaining ILL genes should clarify the role of IAA-conjugate hydrolysis in plant growth and development.

It has been reported that some effects of IAA-amino acids cannot be explained by their hydrolysis to free IAA (Magnus et al., 1992a). Lesions in genes not encoding hydrolases therefore might also result in an IAA-conjugate-insensitive phenotype. For example, the IAA-conjugate-resistant icr1 and icr2 mutants have no defects in hydrolysis of exogenously supplied conjugates (Campanella et al., 1996), and the corresponding mutations map to positions distinct from the six conjugate hydrolase genes described here (Campanella et al., 1996, 1997; Figure 4). We also have identified additional IAA-Ala—insensitive mutants that do not map to known ILL or ICR genes (J. Lasswell, L. Rogg, H. Chen, C. Rongey, M.N. Anderson, and B. Bartel, unpublished data). Analysis of the genes defective in these mutants will provide a more complete picture of IAA-conjugate function and metabolism in plants.

**METHODS**

**Plant Materials and Growth Conditions**

Arabidopsis thaliana ecotypes Columbia (Col-0), Landsberg erecta (Ler), and Wassilewskija (WS) were grown in soil (Metromix 200; Scotts, Marysville, OH) under continuous illumination at 22 to 25°C. Plants were grown aseptically on PNS (plant nutrient medium with 0.5% sucrose; Haughn and Somerville, 1986) solidified with 0.6% agar alone or supplemented with 10 to 100 μM indole-3-acetic acid (IAA) conjugates (from 100-mM stocks in ethanol), 0.05 to 1 μM IAA (from a 10-mM stock in ethanol), or 15 μg/mL kanamycin (from a 25-
mg/mL stock in water). IAA conjugates were obtained from Aldrich (Milwaukee, WI), except IAA-Gly and IAA-Asp, which were obtained from Sigma. Plates were wrapped in gas-permeable Leukopor surgical tape (Beiersdorf Inc., Norwalk, CT) and grown under continuous illumination (25 to 45 μE m⁻² sec⁻¹) with yellow long-pass filters to stabilize indolic compounds (Stasinopoulos and Hangarter, 1990).

Isolation of Mutants

The iar3-1 mutant allele was isolated as follows. WS seeds were mutagenized with ethyl methane sulfonate (EMS), as described previously (Normany et al., 1997). Approximately 24,000 of the resulting M₁ seeds were surface-sterilized (Last and Fink, 1988) and spread at a density of ~1000 seeds per plate on 150 × 25 mm Petri plates containing 100 mL of PNS supplemented with 50 μM IAA-Ala. After 2 weeks, putative mutants with increased root length were transferred to soil and allowed to set seeds. The resultant M₂ seeds were screened separately for resistance to 50 μM IAA-Ala and wild-type sensitivity to 1 μM IAA. The iar3-2, iar3-3, and iar3-4 alleles were similarly isolated from M₂ pools of EMS-mutagenized ilr1-1 seeds on PNS plates supplemented with 100 μM IAA-Ala (300,000 M₂ seeds screened) or 70 μM IAA-Phe (60,000 M₂ seeds screened). The four iar3 alleles failed to complement one another and represented independent alleles based on sequence analysis.

iar3 mutant plants were backcrossed to the WS ecotype before phenotypic analysis. The plants analyzed in Figure 1 had been backcrossed twice, except for the iar3-4 and ilr1-1 iar3-4 plants, which had been backcrossed once. Genotypes at IAR3 and ILR1 were confirmed by polymerase chain reaction (PCR) amplifications of genomic DNA spanning the lesion in each mutant allele and cutting the resultant products with a restriction enzyme that distinguishes between wild-type and mutant alleles (Table 1).

For genetic mapping, DNA was prepared (Celenza et al., 1995) from iar3/iar3 F₂ plants from an outcross to Col-0 and analyzed by PCR with primers that amplify regions polymorphic between these ecotypes (Konieczny and Ausubel, 1993; Bell and Ecker, 1994).

Degenerate PCR

Oligonucleotides were from the Great American Gene Company (Ramona, CA). ILR1-like genes were identified by performing PCR with primers designed from regions similar among ILR1, ILL1, and ILL2 (Figure 3). The forward primer 5′-AARATHCAYGARAAATCCNGA-3′ (48-fold redundancy) was designed to encode the amino acid sequence KHENPE, and the reverse primer 5′-CCYTCTCNCSNCN-GGYTGRAA-3′ (512-fold redundancy; H indicates A, C, or T; N indicates A, C, G, or T; R indicates A or G; S indicates C or G; and Y indicates C or T) was the reverse complement that would encode the sequence FOP(G/A)EEG. These primers were used to amplify Col-0 genomic DNA by PCR by using 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. This resulted in five discrete bands when separated on a 1.5% agarose gel ranging in length from 535 to ~700 bp. These PCR products were gel-purified using the Qiaex II gel extraction kit (Qiagen, Valencia, CA) and TA cloned into pT7Blue (Novagen, Madison, WI). Restriction analysis of the resulting clones identified the known amido-hydroxylase genes IRL3, ILL1, and ILL2. Subclones yielding restriction patterns other than those expected for IRL3, ILL1, and ILL2 were sequenced using T7-primed reactions analyzed on an Applied Biosystems (Foster City, CA) automated DNA sequencer by D. Needleman (University of Texas–Houston Medical School Molecular Genetics Core Facility) or C. Kolenda and J. Derr (Department of Veterinary Pathobiology, Texas A&M University, College Station).

Cloning and Sequencing of IRL1-like Genes

A partial cDNA containing the last three exons of IAR3 (expressed sequence tag [EST] clone 214AAST; Newman et al., 1994) was obtained from the Arabidopsis Biological Research Center (Ohio State University, Columbus). The 5′~650 bp of this clone was cloned after digestion with Smal and used to isolate a full-length IAR3 cDNA by colony hybridization to a plasmid-based cDNA library (Minet et al., 1992) and to isolate the IAR3 genomic DNA by colony hybridization to an Arabidopsis (Col-0 ecotype) cosmid library (Olszewski et al., 1988). The ~5-kb XbaI fragment from an IAR3-positive cosmid was subcloned into pBluescript II KS+ (Stratagene, La Jolla, CA). The IAR3-hybridizing cosmid also contained an ~5.5-kb HindIII fragment that contained the ILL5 gene. This fragment was subcloned into pUC19 for sequencing.

To identify the wild-type (WS) IAR3 sequence and the mutations in the four iar3 mutant alleles, we amplified genomic DNA by PCR by using the following pairs of oligonucleotides: 5′-GCGCAGTTGCTTCTAGTGTCTTTCCCC-3′ and 5′-ATAGAAGCTTACACTGATAAC-3′; and 5′-CCAGCTCCACACTACCTTTGCG-3′ and 5′-CCCCGTCTCATCATCACGCAC-3′. A PCR using 40 cycles of 30 sec at 95°C, 30 sec at 56°C, and 3 min at 72°C yielded a 1135-bp fragment with the first pair of oligonucleotides and a 1033-bp fragment with the second pair. Each PCR product was sequenced directly (Ausubel et al., 1995) by using the primers that were used to amplify the fragment. The base change found in each iar3 mutant allele destroyed a restriction enzyme site (Table 1), allowing us to track the alleles in segregating populations without regard to phenotype by using PCR on genomic DNA followed by restriction digestion.

The region of ILL5 containing the 3′ splice acceptor site for intron two was PCR amplified (using the conditions described above for the iar3 mutant alleles) from genomic Col-0, WS, and Ler DNA by using the forward primer 5′-CCTACGATGCTCTCCTGGTGTCG-3′ and the reverse primer 5′-GATTGGAACATGAGAATTCAGGGG-3′. The resulting 1.2-kb PCR products were sequenced directly (Ausubel et al., 1995) using the forward primer.

The ILL1 and ILL2 genomic DNA was cloned by hybridizing the ILL2 cDNA (Bartel and Fink, 1995) to an Arabidopsis cosmid library (Olszewski et al., 1988). A single cosmid was isolated that contained both genes. This DNA was subcloned into pBluescript II KS+ for sequencing. The ILL3 genomic DNA was similarly cloned from a cosmid isolated by hybridizing the ILL3 cDNA to the cosmid library. A combination of T7- or T3-primed reactions on subclones in pBluescript II KS+ and custom oligonucleotide–primed reactions was sequenced as given above. The genomic sequences of the genes reported here were determined on both strands and have the following GenBank accession numbers: AF047031 for ILL1 and ILL2; AF081066 for ILL3; AF081067 for IAR3; and AF085806 for ILL5.

Genetic Mapping of IRL1-like Genes

The ILR1-like genes were mapped by developing PCR-based polymorphic markers (Konieczny and Ausubel, 1993) and scoring these
markers on DNA prepared from recombinant inbred lines (Lister and Dean, 1993) or F₂ mapping populations. The oligonucleotides and restriction enzymes used were as follows. For ILL1, PCR was with the oligonucleotides 5’-GCATGTGGTGAGCAATGTTGCA-3’ and 5’-TCGAATTGAATTGTCTAGCAA-3’, yielding a 1.3-kb fragment containing three TaqI sites in Col-0 or Ler DNA and four TaqI sites in WS DNA. For ILL2, PCR was with the oligonucleotides 5’-CTCAGTTGCTACTCACTACTTT-3’ and 5’-GCCAATACTTCAACAGAACACATT-3’, yielding a 2.0-kb fragment containing three HaeIII sites in Col-0 DNA and two HaeIII sites in Ler or WS DNA. For ILL3, PCR was with the oligonucleotides 5’-GCGATC-3’ and 5’-CAGAGGCACATGGCTAAGAGG-3’, yielding a 1.2-kb fragment containing one Ndel site in Col-0 DNA and two Ndel sites in Ler or WS DNA. For IAR3, PCR was with the oligonucleotides 5’-GCAACTCAAGAAAAGAAT-3’ and 5’-TTAAGAGAGG-3’, yielding a 126-bp fragment from Col-0 DNA and a 135-bp fragment from WS DNA. For IAR3 protein shown in Figure 3, except for a substitution of an Arg for a Lys, the IAR3 protein is identical to the Col-0 protein, 50 mM Tris, pH 8.0, was diluted into the reaction buffer to a final concentration of 1.7 mg/mL protein, 50 mM Tris, pH 8.0, 1 mM DTT, 1 mM MnCl₂, and 1 mM IAA–amino acid conjugate. To determine the hydrolysis rate of GSTIAR3 with IAA–Phe or IAA–Ile and GSTIar3-1 with IAA–Gly, we used a protein concentration of 3.0 mg/mL. Eight-, 16-, and 24-hr time points were taken for GSTIAR3 or GSTIar3-1 with the IAA–amino acid conjugates indicated, except for GSTIAR3 with IAA–Phe or IAA–Ile and GSTIar3-1 with IAA–Gly, for which 16- and 24-hr time points were taken. For GST alone, one 24-hr time point was taken. At the indicated time points, reactions were stopped by adding 2 volumes of ethanol and were centrifuged by a gradient from 15% to 90% methanol for 10 min followed by a gradient from 15% to 90% methanol for 10 min with 1% acetic acid throughout. Compounds were detected by fluorescence (excitation 278 nm, emission 350 nm; model 1046A programmable fluorescence detector; Hewlett Packard) and quantitated by peak area.

Enzyme Assays
The optimum pH for IAR3 activity was determined at 0.5 pH unit increments by using McIlvaine buffered solutions (Elving et al., 1956) from pH 3.5 to pH 8.0 and 50 mM Tris-buffered solutions from pH 7.5 to pH 9.0. To determine hydrolysis rates, purified GSTIAR3, GSTIar3-1, or GST in 5 mM reduced glutathione in 50 mM Tris, pH 8.0, was diluted into the reaction buffer to a final concentration of 1.7 mg/mL protein, 50 mM Tris, pH 8.0, 1 mM DTT, 1 mM MnCl₂, and 1 mM IAA–amino acid conjugate. The optimum pH for IAR3 was pH 8.0. The substrate solutions were pH 7.5 and 8.0.

RNA Analysis
Total RNA was isolated (Nagy et al., 1988) from mature dry seeds (seeds), roots of 14-day-old plants grown on 3MW gel blot paper (Midwest Scientific) on PNS solidified with 0.6% agar (roots), above-ground parts of 14-day-old plants grown in soil (shoots), stems of 29-day-old plants (stems), siliques of 29-day-old plants (siliques), and buds and flowers of 29-day-old plants (buds) of the Col-0 ecotype. The RNA was analyzed using a NorthernMax kit, according to the recommendations of the manufacturer (Ambion, Austin, TX). An antisense IAR3 RNA probe (Riboprobe in vitro transcription systems; Promega) was used to detect IAR3 mRNA. A 28S rDNA probe was used to verify that equal amounts of RNA were loaded in each lane. The 28S probe was labeled using random oligonucleotides, as described previously (Ausubel et al., 1995), except that 12-mers were used.

Production of Transgenic Plants
To construct the 35S–IAR3 plasmid, we introduced an EcoRI site in the 3’ untranslated region of the IAR3 cDNA by using oligonucleotide-directed mutagenesis (Ausubel et al., 1995) of the pKSIAR3 plasmid with the oligonucleotide 5’-CTGTACCATTTTGAGCAAGACATAGTGCATCATGCG-3’ (altered residues are underlined). The full-length IAR3 cDNA was excised with XbaI and EcoRI and cloned downstream of the cauliflower mosaic virus 35S promoter in

**IAR3 Purification**

A modified glutathione S-transferase (GST) fusion vector was constructed. The complementary oligonucleotides 5’-GATCCCATATGACATTAGCACATTAGCGGACCCCG-3’ and 5’-AATTCCGGAGCCGTCGACC-3’ were synthesized. These oligonucleotides, when annealed, have BamHI and EcoRI overhangs. The annealed oligonucleotides were ligated into pGEX-KOT with BamHI and EcoRI to give pGEX-KOT. The restriction sites present in the multiple cloning site are BamHI, Ndel, Bsp191, Ncol, Sall, NotI, and EcoRI. The full-length IAR3 cDNA (ecotype Ler) was excised from the pFL61 vector (Minet et al., 1992) with NotI and cloned into NotI-cut pBluescript II KS+ to give pKSIAR3. The Ler IAR3 protein is identical to the Col-0 protein shown in Figure 3, except for a substitution of Arg for Lys at position 437. An Ndel site was introduced at codon 19 in the IAR3 cDNA in pKSIAR3 by using oligonucleotide-directed mutagenesis (Ausubel et al., 1995) with the oligonucleotide 5’-GCCAAGGAGGCTA TTACAACAGAACACATT-3’, yielding a 126-bp fragment from Col-0 DNA and a 135-bp fragment from WS DNA that were resolved on a 4% agarose gel.

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the vector pBICaMV (J. Celenza, personal communication) cut with the same enzymes. This construct was introduced into Agrobacterium tumefaciens GV3101 by electroporation (Ausubel et al., 1995). The resultant strain was used to transform Col-0 by vacuum infiltration (Bent et al., 1994). Transformed plants were identified 10 days after germination on PN supplemented with 15 μg/mL kanamycin (Haughn and Somerville, 1986). Homozygous lines were identified in subsequent generations by plating on kanamycin-containing medium. Three independently derived homozygous lines were tested for increased sensitivity to IAA conjugates, and data from one of these lines are shown in Figure 7. The other two lines were similar in phenotype to the iar3 mutant (data not shown), suggesting that the transgene had cosuppressed (Meyer and Saedler, 1996) the endogenous allele.

To construct the 3SS–ILR1 plasmid, we filled in the ends of the NotI fragment containing the full-length ILR1 cDNA (Bartel and Fink, 1995) with the Klenow fragment of DNA polymerase I, and we cloned the resultant fragment into the Smal site of pBICaMV. This construct, in which the ILR1 cDNA is driven in the sense orientation from the 35S promoter, was used to transgenic Col-0 plants, as described above. Four independently derived homozygous lines were tested for increased sensitivity to IAA conjugates, and data from one of these lines are shown in Figure 7.

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