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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 by 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*

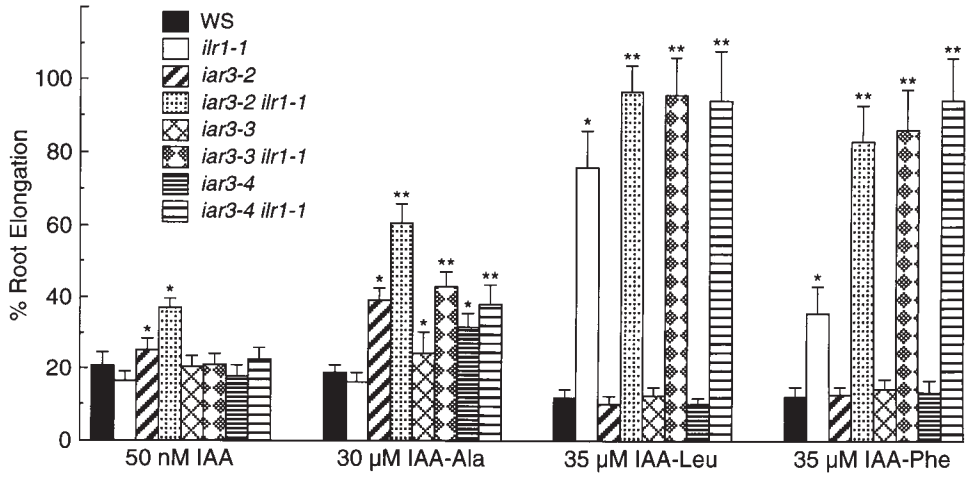

Figure 1. *iar3* Mutants Have Reduced Sensitivity to Certain IAA–Amino Acid Conjugates.

Eight-day-old seedlings grown under yellow-filtered light (Stasinopoulos and Hangarter, 1990) on various media were removed from the agar, and the length of the longest root from each plant was recorded. At least 19 seedlings of each genotype were measured for each condition. Shown is the percentage elongation on IAA, IAA–Ala, IAA–Leu, and IAA–Phe compared with the hormone-free control (see Methods). Error bars indicate the standard deviation of the mean. Single asterisks indicate that measurements were statistically significantly different from that of the wild type (Student's *t* test; $P < 0.001$). Double asterisks indicate that measurements of double mutants were statistically significantly different from those of each single mutant ($P < 0.001$). Differences for all other measurements are not statistically significant ($P > 0.001$).

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 cosmid 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 cosmid, and *IAR3* and *ILL5* were similarly found on a single cosmid, 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* (L*er*) 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 *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).

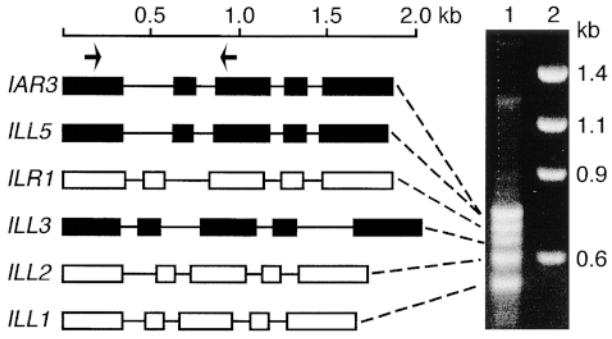

Figure 2. Structure of *IAR3* and Related Genes Isolated By Using Degenerate PCR.

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.

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 PI clone MIK19, 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*

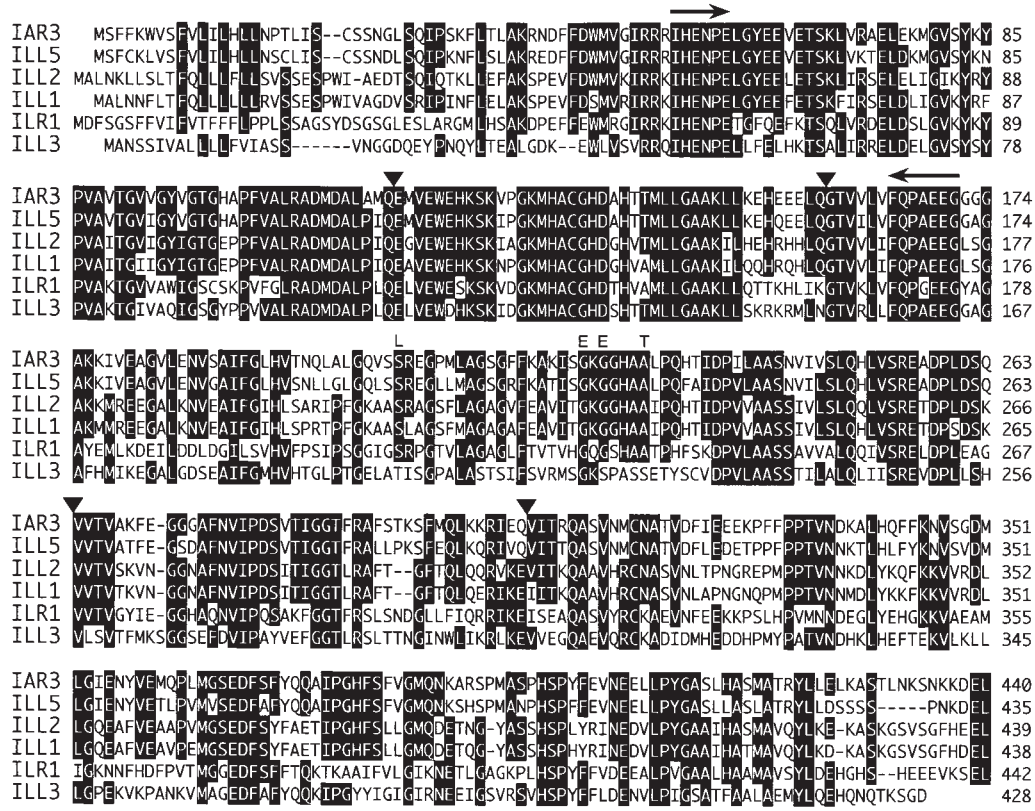

Figure 3. Alignment of Predicted IAA-Conjugate Hydrolases.

Sequences were aligned with the Megalign program (DNAStar, Madison, WI) by using the Clustal method. Amino acid residues identical in at least four of the six sequences are in filled boxes, and hyphens indicate gaps introduced to maximize alignment. The amino acids altered in the four *iar3* alleles are indicated above the wild-type sequence. Triangles indicate positions of introns, and arrows mark the regions used to design degenerate PCR primers. Sequences shown are from the Col-0 ecotype.

gene. The *IAR3* coding region in WS had five single-base differences from the Col-0 sequence, of which only one altered an amino acid (Lys to Arg at position 437).

IAR3 Is an IAA–Ala Hydrolase

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.

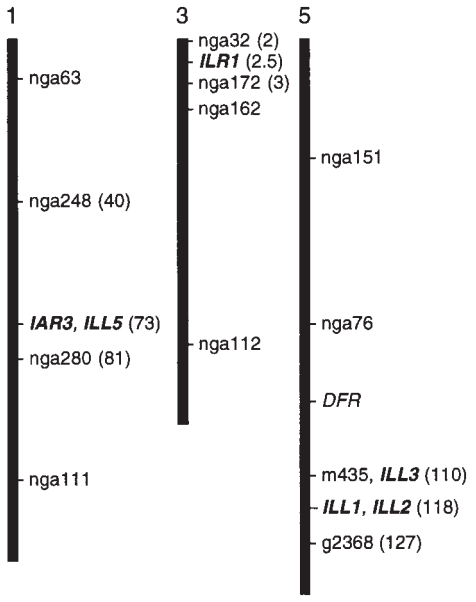

Figure 4. Map Positions of *ILR1*-like Genes.

The *ILR1*-like genes were mapped to chromosomes 1 and 5, as described in Methods. *ILR1* was previously shown to map to the top of chromosome 3 (Bartel and Fink, 1995). Marker positions are from the February 1998 version (http://genome-www.stanford.edu/Arabidopsis/ww/Feb98RImaps/index.html) of a Col-0/L*er* recombinant inbred map (Lister and Dean, 1993). Approximate map positions (in centimorgans) of the *ILR1*-like genes and flanking markers are shown within parentheses.

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

Table 1. Mutations Found in *iar3* Alleles

Allele	Mutagen	Codon Change[a]	Amino Acid Change	Restriction Enzyme Site Change
iar3-1	EMS	TCG→TTG	Ser-106→Leu	Destroys XhoI
iar3-2	EMS	GGG→GAG	Gly-124→Glu	Destroys AciI
iar3-3	EMS	GCT→ACT	Ala-130→Thr	Destroys AluI
iar3-4	EMS	GGA→GAA	Gly-126→Glu	Destroys MnlI

[a]Nucleotide changes are underlined.

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).

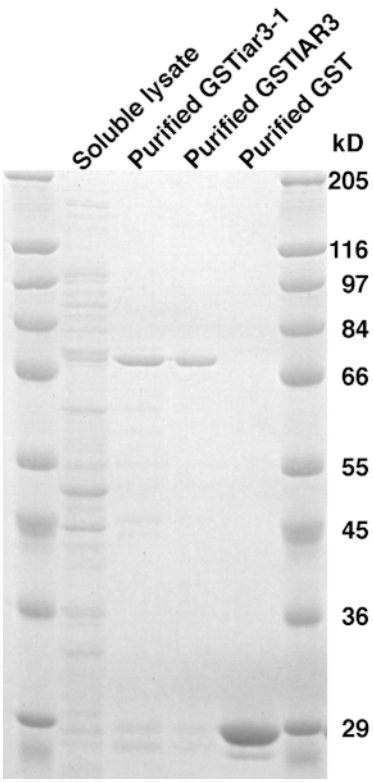

Figure 5. Purified Fusion Proteins Used in Activity Assays.

Purified GST, GSTIAR3, and GSTiar3-1 (see Methods) are shown next to the GSTiar3-1 lysate and molecular mass standards (given at right in kilodaltons; Wide Range Sigmamarkers; Sigma). Twenty micrograms of total protein was electrophoresed on a 9% SDS–polyacrylamide gel and stained with Coomassie Brilliant Blue R 250 (Sigma) (Ausubel 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). This wound- and JA-induced expression is blocked in the JA-insensitive mutant *coi1* and is mediated by a phosphorylation-dependent signal transduction pathway (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

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 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

	GSTIAR3[a]		GSTiar3-1[a]	
Substrate	Hydrolase Activity (IAA released; pmol min^{-1} mg^{-1} protein)	% of Maximum Activity	Hydrolase 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	—

[a]Rates 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.

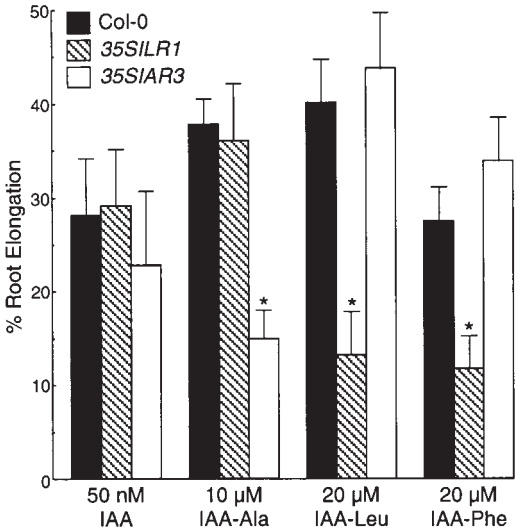

Figure 7. Overexpression of IAA-Conjugate Hydrolases in Transgenic Plants Increases Sensitivity to IAA–Amino Acid Conjugates.

Root elongation of 8-day-old seedlings was quantified as given in the legend to Figure 1, except that 10 or 20 μM IAA conjugates was used to allow detection of increased sensitivity. Asterisks indicate that measurements were statistically significantly different from those of the wild type (Student's *t* test; $P < 0.001$). Differences for all other measurements are not statistically significant ($P > 0.001$).

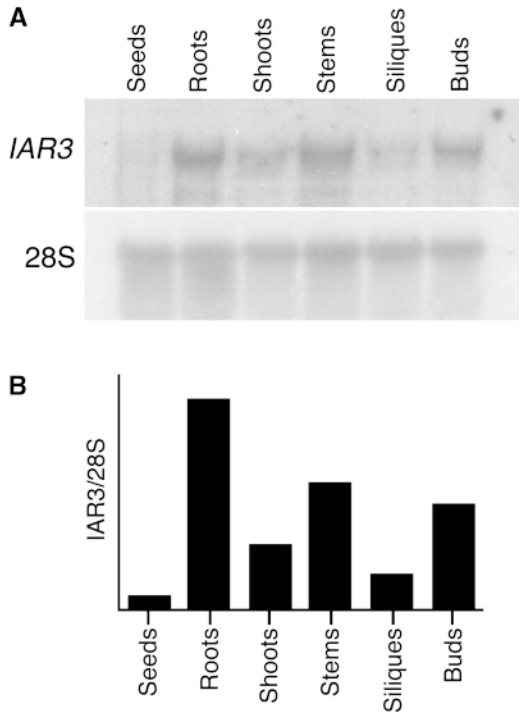

Figure 6. Tissue Specificity of *IAR3* Expression.

Four micrograms of total RNA prepared from different tissues of the ecotype Col-0 was separated on 1% agarose gels and transferred to nylon membranes, as described in Methods.

(A) Hybridization of the membrane with an antisense *IAR3* RNA probe was used to detect *IAR3* mRNA. The 28S probe was used as a loading control.

(B) A PhosphorImager (Fujix BAS1000; Fuji, Tokyo) was used to quantify the amount of signal in each band, and the graph represents the *IAR3* signal divided by the 28S signal in arbitrary units.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotypes Columbia (Col-0), Landsberg *erecta* (L*er*), 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 methanesulfonate (EMS), as described previously (Normanly et al., 1997). Approximately 24,000 of the resulting M_2 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 M_3 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 M_2 pools of EMS-mutagenized *ilr1-1* seeds on PNS plates supplemented with 100 μM IAA–Leu (300,000 M_2 seeds screened) or 70 μM IAA–Phe (60,000 M_2 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* F_2 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′-AARATHCAYGARAATCCNGA-3′ (48-fold redundancy) was designed to encode the amino acid sequence KIHENPE, and the reverse primer 5′-CCYTCYTCNSCNGGYTGRAA-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 FQP(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 amidohydrolase genes *ILR1*, *ILL1*, and *ILL2.* Subclones yielding restriction patterns other than those expected for *ILR1*, *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 *ILR1*-like Genes

A partial cDNA containing the last three exons of *IAR3* (expressed sequence tag [EST] clone 214A5T7; Newman et al., 1994) was obtained from the Arabidopsis Biological Research Center (Ohio State University, Columbus). The 5′ ~650 bp of this clone was isolated after digestion with SmaI 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′-GGGCACAGTGGTTCTAGTTTTCC-3′and 5′-ATAGAAGCTTGACACTGCATAAC-3′; and 5′-CCAGCCTCCACAATCTTCTTTGC-3′ and 5′-CCCCTGCTTCATCATCACGCAC-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 L*er* DNA by using the forward primer 5′-CACTACGATGCTCCTCGGTGCTGC-3′ and the reverse primer 5′-GATTGACAACTGAGAATTACAGGG-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 *ILR1*-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 F_2 mapping populations. The oligonucleotides and restriction enzymes used were as follows. For *ILL1*, PCR was with the oligonucleotides 5′-GCATGCTTGTGGACATGATGGTCA-3′ and 5′-TCGCAATTGAAATTGTTCTACGAA-3′, yielding a 1.3-kb fragment containing three TaqI sites in Col-0 or L*er* DNA and four TaqI sites in WS DNA. For *ILL2*, PCR was with the oligonucleotides 5′-CTCAGTTTGACTTTCCAACTACTCCTTT-3′ and 5′-GCCAATACATTACAACAAGACCAATG-3′, yielding a 2.0-kb fragment containing three HaeIII sites in Col-0 DNA and four HaeIII sites in L*er* or WS DNA. For *ILL3*, PCR was with the oligonucleotides 5′-GTCAACAAAGTCAATTTTCCGATC-3′ and 5′-CAGAGAGCACATGGCTTAAGAGAGG-3′, yielding a 1.2-kb fragment containing one NdeI site in Col-0 DNA and two NdeI sites in L*er* or WS DNA. For *IAR3*, PCR was with the oligonucleotides 5′-GCAACTCAAGAAAAGAATTGAGC-3′ and 5′-GCTCGCTTGCCTTGTGATAACCTG-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.

IAR3 Purification

A modified glutathione *S*-transferase (*GST*) fusion vector was constructed. The complementary oligonucleotides 5′-GATCCCATATGACCATGGCTGTCGACCTCGAGGCGGCCGCG-3′ and 5′-AATTCGCGGCCGCCTCGAGGTCGACAGCCATGGTCATATGG-3′ were synthesized. These oligonucleotides, when annealed, have BamHI and EcoRI overhangs. The annealed oligonucleotides were ligated into pGEX-KT (Hakes and Dixon, 1992) cut with BamHI and EcoRI to give pGEX-KTO. The restriction sites present in the new multiple cloning site are BamHI, NdeI, Bsp191, NcoI, SalI, NotI, and EcoRI. The full-length *IAR3* cDNA (ecotype L*er*) was excised from the pFL61 vector (Minet et al., 1992) with NotI and cloned into NotI-cut pBluescript II KS+ to give pKSIAR3. The L*er* IAR3 protein is identical to the Col-0 protein shown in Figure 3, except for a substitution of an Arg for a Lys at position 437. An NdeI 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′-CCCATTAGAGGAACATGAAATCATATGCGGATTAAGCAAGTGAAGG-3′ (altered residues are underlined). The resultant *IAR3* cDNA was excised with NdeI and NotI and inserted in frame with GST in pGEX-KTO cut with the same enzymes to give pGEX-IAR3, which encodes GST fused to the IAR3 protein (GSTIAR3) with the putative signal sequence (amino acid residues 1 to 19) deleted. To obtain the iar3-1 protein, we mutagenized the *IAR3* cDNA by using oligonucleotide-directed mutagenesis (Ausubel et al., 1995) with the oligonucleotide 5′-CAACATTGGACCCTCTCTCAAGCTCACTTGACCTAATGCC-3′ (altered residue is underlined). The 650-bp MfeI-NcoI fragment of the *iar3-1* cDNA was then inserted into the pGEX-IAR3 plasmid cut with MfeI and NcoI to give the pGEX–iar3-1 plasmid, which encodes GSTiar3-1. The IAR3-coding regions of pGEX-IAR3 and pGEX–iar3-1 were verified by sequencing.

Protein was expressed in DH5α, as described previously (Ausubel et al., 1995), with several modifications. A 100-mL overnight culture (Luria-Bertani medium supplemented with 100 μg/mL ampicillin) was diluted into 400 mL of the same medium and allowed to grow for 1.5 hr at 28°C before induction with 50 μM isopropyl β-D-thiogalactopyranoside. After 3 hr at 28°C, cells were harvested by centrifugation and lysed by sonication, and the soluble lysate was incubated with glutathione–agarose (Sigma) overnight at 4°C. Fusion proteins were eluted with 5 mM reduced glutathione in 50 mM Tris, pH 8.0, for 3 hr at 4°C. Purified protein fusions were quantitated using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL) and diluted to 3.3 mg/mL in 50 mM Tris, pH 8.0, with 5 mM reduced glutathione.

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 $MnCl_2$, 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 filtered by centrifuging through a 0.2-μm microspin filter unit (Midwest Scientific, St. Louis, MO). These samples (10 to 30 μL) were analyzed by HPLC (model 1100 series binary pump; Hewlett Packard, Wilmington, DE) with a Zorbax C-18 column (4.6 × 250 mm; Dupont, Wilmington, DE) at 2 mL/min. The solvent system was 15% 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.

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), aboveground 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. 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′-CTGTTACCATGTTTTGACAGAATTCAGAGTAGAAGAATAGG-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

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 35S–*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 SmaI site of pBICaMV. This construct, in which the *ILR1* cDNA is driven in the sense orientation from the 35S promoter, was used to make 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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