Characterization of a Family of IAA-Amino Acid Conjugate Hydrolases from Arabidopsis*

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The mechanisms by which plants regulate levels of the phytohormone indole-3-acetic acid (IAA) are complex and not fully understood. One level of regulation appears to be the synthesis and hydrolysis of IAA conjugates, which function in both the permanent inactivation and temporary storage of auxin. Similar to free IAA, certain IAA-amino acid conjugates inhibit root elongation. We have tested the ability of 19 IAA-L-amino acid conjugates to inhibit Arabidopsis seedling root growth. We have also determined the ability of purified glutathione S-transferase (GST) fusions of four Arabidopsis IAA-amino acid hydrolases (ILR1, IAR3, ILL1, and ILL2) to release free IAA by cleaving these conjugates. Each hydrolase cleaves a subset of IAA-amino acid conjugates in vitro, and GST-ILR1, GST-IAR3, and GST-ILL2 have values that suggest physiological relevance. In vivo inhibition of root elongation correlates with in vitro hydrolysis rates for each conjugate, suggesting that the identified hydrolases generate the bioactivity of the conjugates.

Indole-3-acetic acid (IAA)

producing free IAA. IAA-Ala is hydrolyzed by extracts of Chinese cabbage (4), by pea stem segments (5), and by purified GST-IAR3 enzyme (6). This hydrolysis correlates with IAA-Ala activity in bioassays. For example, the curvature generated by IAA-Ala applied to bean stem sections is directly related to the measured amount of IAA released from IAA-Ala (7). Similarly, IAA-Leu, IAA-Phe, IAA-glucose, and IAA-myo-inositol are active in bioassays and are hydrolyzed by plant extracts and/or purified enzymes (4, 6, 8–10). Hydrolyzable conjugates probably provide a readily accessible storage form of IAA. For example, IAA conjugates stored in seeds are rapidly hydrolyzed following imbibition to yield the IAA necessary for germination (11, 12).

In contrast, other conjugates such as IAA-Asp can be intermediates in the oxidative degradation of IAA and are not hydrolyzed in vivo (13, 14). Although the plant enzymes that synthesize IAA-amino acid conjugates have not been identified, IAA levels apparently regulate which conjugates are formed. For example, the auxin-overproducing mutant sur2 accumulates more IAA-Asp than wild type (15) but synthesizes less of the hydrolyzable IAA-Leu (8) from applied IAA (15). Certain IAA-amino acid conjugates have activities that are only partially explained by hydrolysis (to release IAA), suggesting that conjugates may have hydrolysis-independent functions. For example, pretreating tomato cell cultures with IAA-Ala inhibits IAA-induced shoot growth and root initiation, suggesting that IAA and some conjugates may compete for a binding site(s) (16).

Several conjugates have been identified and quantified in plants, including IAA-myo-inositol, IAA-glucose, and a large ester-linked cellulosic glucan conjugate in maize (17); IAA-Asp and IAA-Glu in soybean (18, 19); several IAA-peptide conjugates in bean (20, 21); and IAA-Ala in Picea abies (14). IAA-glucose, IAA-Asp, IAA-Glu (22), IAA-Ala, IAA-Leu (23), and an IAA-peptide (21) are present in Arabidopsis.

Plant genes involved in synthesizing ester-linked IAA conjugates and hydrolyzing amide-linked IAA conjugates have been identified. Iaglu from maize (24) and UGT84B1 from Arabidopsis (25) encode enzymes that esterify IAA to glucose. ILR1 and IAR3 encode IAA-amino acid hydrolases that were identified through mutant screens in Arabidopsis. ilr1 and iar3 mutant plants are less sensitive than wild type to IAA-Leu and IAA-Ala, respectively (6, 8), and the ILR1 and IAR3 proteins are 46% identical to each other. Five additional Arabidopsis amidohydrolase-like genes have been identified based on homology (Fig. 1). ILL1 and ILL2 (8) are 87% identical to each other, ~57% identical to IAR3, and ~44% identical to ILR1. ILL3 is 42–48% identical to other hydrolases, and ILL5 is an apparent pseudogene most similar to IAR3 (6). A genomic sequence recently deposited in GenBank (G1; GenBank accession number CAAn9330) potentially encodes a protein...
that we refer to as ILL6 (cDNA GenBank™ accession number AY065996) that is 45–48% identical to the other hydrolases. All of the Arabidopsis amidohydrolase-like proteins contain N-terminal sequences predicted to target insertion into the endoplasmic reticulum (ER), and all except ILL3 and ILL6/GR1 encode C-terminal putative ER retention signals, suggesting that most family members localize to the ER lumen (6, 8). Although these Arabidopsis hydrolases lack close homologs in non-plant eukaryotes, they resemble (19–34% identical) microbial hydrolases (Fig. 1) that accept diverse substrates, including IAA-Asp (26), acetylated amino acids (27), benzoylcarbonyl-modified amino acids (28), and benzoylglycine (29).

One measure of IAA-amino acid conjugate activity is the ability of certain conjugates to inhibit Arabidopsis root elongation (6, 8, 30, 31). Because the profile of endogenous IAA conjugates is still incomplete for Arabidopsis and most other plants, we wished to determine which IAA-amino acid conjugates display auxin-like activity in this bioassay and to determine whether auxin activity correlates with the ability of purified amidohydrolases to cleave these conjugates. Here we describe the synthesis of 13 IAA-l-amino acid conjugates and compare the activities of 19 IAA-l-amino acid conjugates on wild type and mutant Arabidopsis root elongation. We also describe purification of GST fusions of the amidohydrolases ILR1, ILR3, ILL1, and ILL2 and compare the in vitro enzymatic activity of these hydrolases, including rates of hydrolysis, pH optima, cofactor requirements, and kinetics.

**EXPERIMENTAL PROCEDURES**

**Synthesis of IAA-Amino Acid Conjugates—**IAA-Ala, IAA-Asp, IAA-Ile, IAA-Leu, IAA-Phe, and IAA-Val were purchased from Aldrich; other chemicals were purchased from Sigma. The remaining conjugates were synthesized by dicyclohexylcarbodiimide (DCCI)-activated condensation (32) of IAA and l-amino acid esters, followed by base saponification to remove ester-protecting groups and yield IAA-amino acid conjugates. Protected l-amino acids used were methyl esters for Gly, His, Met, Pro, Ser, Thr, and Tyr; dimethyl esters for cystine and Glu; t-butyrl esters for Asn and Gln; N-Cbz esters for Lys; and N,N-di-Cbz ester for Arg. IAA, the protected amino acid ester, triethylamine, and DCCI were dissolved in methylene chloride and incubated at room temperature with stirring. When TLC monitoring determined that reactions had gone to completion, they were quenched by adding water to a final concentration of 10%. Dicyclohexylurea was removed by gravity filtration; reactions were dried in vacuo, and products were dissolved in 10% methanol in chloroform. The protected IAA conjugates were then purified on a silica gel column using a solvent system of 10% methanol in chloroform. Pure fractions were combined, dried in vacuo, and dissolved in methanol. Conjugates were deprotected by KOH saponification at room temperature, except for IAA-Pro, which was deprotected in formic acid at 37 °C. Because IAA-Arg did not readily deprotect but yielded an array of products, the synthesis of this conjugate was abandoned. Deprotected conjugates were purified on a silica gel column using a solvent system of 50% 2-butanol, 30% ethyl acetate, 10% ethanol, and 10% water (33). Fractions containing the pure amino acid conjugate were further purified by reverse phase HPLC on a Phenomenex Prodigy 10 μm ODS preparative 250 × 21.20-mm column in a solvent system of 50% methanol in 1% acetic acid with a flow rate of 4 mL/min. Pure fractions were collected using a Waters Jr. fraction collector (ISCO, Lincoln, NE), combined, dried in vacuo, and stored at −20 °C. Before use, conjugates were dissolved in 50 or 100% ethanol to a stock concentration of 20 to 100 mM, depending on solubility.

**IAA-Amino Acid Conjugate Bioassay—**Conjugates, from 20 to 100 mM stocks in 50 or 100% ethanol, were added to plant nutrient medium including 0.5% sucrose (PNS) (34) solidified with 0.6% agar. Arabidopsis seeds from the Wassilewskija accession (wild type), ilr1–1 (8), iar3–2 (6), and ilr1–1 iar3–2 were surface-sterilized and plated on PNS medium containing 40 μM IAA-amino acid. Plates were incubated at 22 °C under yellow long-pass filters (35) with constant illumination (25–45 μE m⁻² s⁻¹). After 8 days, plants were removed from the agar, primary root lengths were measured, and the average root length was calculated.

**Generation and Purification of GST Fusion Proteins—**The amidohydrolases (without the predicted N-terminal signal sequences) were expressed in Escherichia coli as fusions to the C terminus of GST. pGEX-ILR1 was made by introducing an NdeI site at codons 22–23 in the ILR1 cDNA (8) and subcloning the NdeI-EcoRI fragment into pGEX-KTO (6) cut with the same enzymes. pGEX-ILL1 and pGEX-ILL2 encode GST fused to the ILL1 or ILL2 cDNAs (8) at codon 23 or 25, respectively. pGEX-IAR3 was previously described (6).

**FIG. 1.** Phylogenetic tree of amidohydrolase homologs from plants and microorganisms. A, percent identity between Arabidopsis (At) IAA conjugate hydrolases, ILR1, ILL1, ILL2 (8), and IAR3 (6); related Arabidopsis proteins ILL5, ILL6, and ILL6 (GenBank™ accession number AY065986); and the microbial proteins E. agglomerans (Ea) IaasPH (26), Bacillus stearothermophilus (Bs) Ama (27), Sulfolobus solfataricus (Ss) CpsA (28), Campylobacter jejuni (Cj) HipO (29), and the E. coli (Ec) protein AbgA (41). Complete predicted protein sequences were aligned with the MegAlign program (DNAStar) using the ClustalV method, and the percent identities of the various pairs are shown. B, the tree reconstructs the evolutionary relationships among IAA-conjugate hydrolases and related proteins from plants and microbes. Sequences shown in panel A were aligned with homologs uncovered by scanning and assembling genome project information from various plants. Some of these plant homologs (found in the Institute for Genomic Research (TIGR) website www.tigr.org) were: Lycopersicon esculentum (Le) TC58165 and TC6219, Medicago truncatula (Mt) TC36030 and TC33457, Oryza sativa (Os) TC48523, and Solanum tuberosum (St) TC18857. Other homologs were manually assembled from partial cDNA sequences in GenBank™. The GenBank™ accession numbers of the 5'-cDNA of each contiguous alignment are: Glycine max (Gm): 1, BG042742, and 2, AW234471; Gossypium hirsutum (Gh): 1, BF728001; Triticum aestivum (Ta): 1, BE471246; and Mt: 1, AW868159, and 2, BF641477. Sequences corresponding to amino acids 39–200 in ILR1 were aligned with the MegAlign program (DNAStar) using the ClustalV method, and the unrooted phylogram was generated by using PAUP.
For protein expression, single colonies from freshly transformed BL21 (DE3) E. coli cells were inoculated into 100 ml of Luria broth containing 100 µg/ml ampicillin and grown for 6–8 h at 37 °C. Cultures were allowed to cool to room temperature, induced by adding isopropyl-1-thio-β-D-galactopyranoside to 50 µM and grown overnight with shaking at 18 °C. The proteins were purified as described (6) and stored at −80 °C until analysis. Proteins were quantified using SDS-PAGE and Coomassie Blue staining next to bovine serum albumin standards of known concentration.

To assess whether the GST fusion proteins were similarly folded, buffer was exchanged into 10 mM sodium phosphate buffer, pH 7.6, and protein concentrations were quantified using Microcoulometric centrifugal filter devices (Millipore, Bedford, MA). Circular dichroism spectra were collected on an AVIV model 62A DS circular dichroism spectrometer.

**Determination of Hydrolysis Rates**—For preliminary activity assays, reactions contained 50 mM Tris, pH 8.0, 1 mM dithiothreitol, 1 mM MnCl₂, 1 mM IAA-amino acid, and between 2 and 20 ng/µl GST fusion protein. Reactions were incubated at room temperature for 16–24 h. To determine whether any IAA was released from the conjugate, reaction products were separated by TLC in ethyl acetate/2-butanone/ethanol/water (5:3:1:1) (36), then stained with Van Urk-Salkowski reagent to visualize indolic compounds (33). Under these conditions GST-ILR1, GST-IAR3, and GST-ILL2 released IAA from one or more conjugates at a measurable rate. GST-ILR1, GST-IAR3, and GST-ILL2 reactions were diluted in 65 µl of hydrolysis buffer (50 mM Tris (pH 7.5 for GST-ILR1 and GST-ILL1 or pH 8.0 for GST-ILL2 and GST-IAR3), 1 mM dithiothreitol, 1 mM MnCl₂, 1 mM IAA-amino acid) to a final concentration of 15 µg/µl for GST-IAR3 and GST-ILL2, 10 ng/µl for GST-ILL1, 100 ng/µl for GST-ILL1, and 15 µg/µl for GST. All assays were performed at room temperature. At each time point, 15 °C of the reaction was stopped by dilution in 135 µl of methanol with 1% acetic acid and stored at −20 °C until HPLC analysis. For GST-ILR1 and GST-ILL1, time points were taken at 1, 3, 5, and 20 h for all conjugates. For GST-ILL2, time points were taken at 15, 30, 45, and 60 min. For GST-IAR3, time points were taken at 2, 3, and 5 h for IAA-Ala, -Asp, -Gly, -Ser, -Thr, -Glu, -His, -Lys, -Asn, and -Gln; 4, 6, and 24 h for IAA-Phe, -Ile, -Leu, and -Trp; 2, 4, and 7 h for IAA-Glu,-His,-Lys,-Asn, and -Gln; and 2, 5, and 7 h for IAA-Cys,-Met,-Pro,-Val,- and -Tyr.

**HPLC Analysis**—The percentage of IAA released from each conjugate was determined using HPLC. Following centrifugation to pellet insoluble material, 100 µl of each sample was injected on a Phenomenex Ultracarb 5µ OD300 50 × 4.6-mm reverse phase column. Flow rate was either 2 or 3 ml/min. The solvent system was 1% acetic acid in 20% methanol for IAA-Ala, -Asp, -Gly, -Ser, -Thr, -Glu, -His, -Lys, -Asn, and -Gln; 1% acetic acid in 50% methanol for IAA-Phe, -Ile, -Leu, and -Trp; a gradient from 20 to 100% methanol over 25 min with 1% acetic acid throughout for IAA-Cys, -Met, -Pro, -Val, and -Tyr; or a gradient from 15 to 100% methanol over 25 min for Kₐ, metal cofactor, and pH experiments. Rates were calculated as nanomoles of IAA released per minute per milligram of fusion protein for each time point.

**Kinetic Analysis**—For cofactor optimization, reactions contained 1 mM MnCl₂, 1 mM dithiothreitol, and 1 mM IAA-Ala plus either 50 mM HEPES (pH 6.5, 7.0, or 7.5) or 50 mM Tris-HCl (pH 7.5, 8.0, or 8.5). Protein concentrations for the purified enzymes were the same as for the rate determination, and reaction volumes were 50 µl. For GST-ILL1, GST-IAR3, and GST-ILL1, reactions were stopped as described above at 2.5 h. For GST-ILL2, reactions were stopped at 10 min. Released IAA was measured by HPLC as described above.

**Metal Cofactor Optimization**—For cofactor optimization, reactions contained 1 mM dithiothreitol, 1 mM IAA-Ala, and either 50 mM Tris, pH 7.5, for GST-ILL1 or 50 mM Tris, pH 8.0, for GST-ILL2 and GST-IAR3. For GST-ILL1, reactions contained 1 mM dithiothreitol, 1 mM IAA-Phe, and 50 mM Tris, pH 7.5. Metal cofactors or EDTA was added to a 1 mM final concentration from 10 mM stocks in water of ZnSO₄, CuSO₄, MnCl₂, CoCl₂, CaCl₂, MgCl₂, or EDTA. Reactions were stopped as described above at 10 or 20 min for GST-ILL2 and at 2.5 or 4 h for the other proteins. IAA released was quantified by HPLC as described above.

**Results**

**Analysis of IAA-Amino Acid Conjugate Effects on Root Growth**—To systematically analyze the Arabidopsis amidohydrolase family, we first synthesized and purified 13 IAA-amino acid conjugates that are not commercially available (see “Experimental Procedures”). Wild type, iar1, iar3, and iar1 iar3 seedlings were then grown on medium containing 40 µM IAA-amino acid conjugate for 8 days, and mean root lengths were compared with lengths on unsupplemented medium (Fig. 2). In addition to the previously reported IAA-Ala, -Leu, and -Phe (6, 8), IAA-t-amino acid conjugates that efficiently inhibited wild type root elongation include IAA-Asn, -Gln, -Glu, -Gly, -Met, -Ser, -Thr and -Tyr, all of which inhibited root elongation by greater than fifty percent at 40 µM. In contrast, other conjugates did not efficiently inhibit root elongation, such as IAA-Asp, -Cys, -His, -Ile, -Lys, -Pro, -Thr, and -Val, which inhibited elongation by less than fifty percent at 40 µM. As previously shown with IAA-Ala, IAA-Leu, and IAA-Phe (6), the hydrolase mutants iar1-1 and iar3-2 were less sensitive to the inhibitory effects of many bioactive conjugates, and the double mutant iar1-1 iar3-2 was less sensitive to all of the bioactive conjugates (Fig. 2). This suggests that at least part of the auxin activity of these conjugates requires hydrolysis to release free IAA. Inactive conjugates might either be poor substrates of the hydrolases or enter plant cells inefficiently. To distinguish between these possibilities, we examined the in vitro substrate specificities of the amidohydrolases.

**Analysis of IAA-Amino Acid Conjugate Hydrolysis**—Extracts from E. coli expressing IRL1 acquire the ability to hydrolyze IAA-Phe and IAA-Leu (8), and a purified GST-IAR3 fusion hydrolyzes IAA-Ala (6). To extend this analysis to include kinematic parameters, additional conjugates, and additional enzymes, we extracted four of the Arabidopsis amidohydrolases as fusions to GST and purified the recombinant proteins (see “Experimental Procedures”). Circular dichroism spectroscopy suggested that all four purified fusion proteins were similarly folded (data not shown). In addition to GST-ILR1 and GST-IAR3, GST-ILL1 and GST-ILL2 hydrolyzed a subset of the IAA-amino acid conjugates. We therefore undertook a systematic comparison of GST-ILR1, GST-IAR3, GST-ILL1, and GST-ILL2 with the 19 IAA-amino acid conjugates.

**Determination of Amidohydrolase pH and Cofactor Optima**—For pH optimization, purified GST-hydrolase fusions were incubated with IAA-Ala in HEPES- or Tris-buffered reactions, and the rate of hydrolysis for each was determined by HPLC quantification of IAA released from the conjugate (see “Experimental Procedures”). As shown in Table I, GST-ILL2, like GST-IAR3 (6), was most active at pH 8, whereas GST-ILR1 and GST-ILL1 were more active at pH 7.5. All the hydrolases preferred Tris buffer to HEPES. For metal ion cofactor analysis, GST-hydrolase fusions were incubated with IAA-Ala or IAA-Phe in Tris-buffered reactions with ZnSO₄, CuSO₄, MnCl₂, CoCl₂, CaCl₂, MgCl₂, or EDTA, and hydrolysis rates were calculated. As shown in Table II, all hydrolases prefer manganese over cobalt, and IRL1 could also utilize copper. Cobalt supported some activity with all hydrolases but precipitated out of solution in the presence of dithiothreitol and is therefore not shown.

**Determination of Substrate Specificities**—To compare the substrate specificities of the enzymes, purified GST fusions of each amidohydrolase were incubated with each of 19 IAA-t-amino acid conjugates. The amount of IAA liberated from
conjugate was measured by HPLC at three or more time points, and the average hydrolysis rates are shown in Table III. Similarly purified and incubated GST did not detectably hydrolyze any of the conjugates (data not shown). All four hydrolases cleaved several IAA-amino acid conjugates, including IAA-Ala. IAA-Phe was the best substrate of GST-ILR1, whereas GST-ILL2 and GST-IAR3 cleaved IAA-Ala most efficiently. GST-ILL1 hydrolysis was restricted to IAA-Ala, IAA-Asn, and IAA-Tyr, which were all cleaved at similar rates. Interestingly, GST-ILL2 hydrolyzed its preferred substrate, IAA-Ala, at least 60-fold faster than any of the other hydrolases. Although they hydrolyze more slowly, ILR1 and IAR3 clearly contribute to in vivo IAA-amino acid conjugate hydrolysis, because loss-of-function mutations in these enzymes confer resistance to exogenous conjugates (Fig. 2) (6, 8).

**Kinetic Analysis of the Amidohydrolases**—To determine the possible relevance of the observed hydrolysis, we determined the $K_m$ for each hydrolase on IAA-Ala and IAA-Leu, two amino acid conjugates that have auxin activity (Fig. 2) (6, 8) and are found endogenously in Arabidopsis seedlings (23). Purified GST fusions of ILR1, IAR3, ILL1, and ILL2 were incubated with varying concentrations of IAA-Ala or IAA-Leu, and the reaction rate was determined at each concentration (Fig. 3). $V_{max}$ and $K_m$ values were calculated from these data (Table IV). GST-ILL2 has the highest $V_{max}$ on both IAA-Ala and IAA-Leu. GST-ILR1 and GST-IAR3 both show lower but significant $V_{max}$ values, and GST-ILR1, GST-IAR3, and GST-ILL2 have $K_m$ values within an order of magnitude of each other. It is interesting to note that GST-ILL1 has the lowest $K_m$ values. In contrast, GST-ILL1 shows little activity and has a $K_m$ 3–30 times higher than the other enzymes. Because GST-ILR1, -IAR3, and -ILL2 all have $K_m$ values in the micromolar range, these enzymes are probably physiologically relevant. Calculating the catalytic efficiency ($k_{cat}/K_m$) reveals that GST-ILL2 is the most efficient enzyme on IAA-Ala, whereas ILR1 is the most efficient on IAA-Leu (Table IV). This is particularly interesting when considering the recent finding that IAA-Ala and IAA-Leu are distributed in different tissues of Arabidopsis seedlings (23).

**DISCUSSION**

ILR1 and IAR3 encode IAA-amino acid hydrolases, and Arabidopsis has five additional amidohydrolase-like genes (Fig. 1). ILL5 is apparently a pseudogene (6), and we have not found conditions in which GST fusions of ILL3 or ILL6 hydrolyze IAA-amino acids.2 Here we have shown that the two remaining ILR1-like genes, ILL1 and ILL2, each encode IAA-amino acid hydrolases, and we have determined the substrate specificities and kinetic parameters of all four Arabidopsis IAA-amino acid hydrolases. In addition, we have determined the biological activity of 19 IAA-amino acid conjugates on wild type Arabidopsis root elongation. Conjugates that are highly active in this bioassay are substrates of at least one GST-amidohydrolase fusion (Fig. 2 and Table III). Moreover, the ilr1 iar3 double mutant lacks two amidohydrolases and is less sensitive than wild type to all biologically active conjugates (Fig. 2), suggesting that ILR1 and IAR3 hydrolyze these conjugates in vivo. In contrast, the conjugates that lack significant activity in root elongation inhibition assays are generally poor substrates of all of the amidohydrolases, consistent with the hypothesis that conjugate bioactivity derives from hydrolysis (Fig. 2 and Table III).

Comparing hydrolysis rates, GST-ILL2 appears more active than the other amidohydrolases, suggesting that ILL2 also may be an important IAA-amino acid hydrolase in vivo. GST-ILL2 also shows the broadest range of substrate specificity (Table III). However, comparing the catalytic efficiencies ($k_{cat}/K_m$) reveals that whereas ILL2 is the most efficient enzyme for IAA-Ala, ILR1 is the most efficient enzyme for IAA-Leu (Table IV). Although ill2 mutants have not been isolated through classical genetic screens, the advent of facile reverse genetic methods (37) may allow us to test the importance of ILL2 in Arabidopsis development and compare the relative activities of ILR1 and ILL2 in vivo. Although GST-IAR3 is less efficient than GST-ILL2 on IAA-Ala, it clearly can hydrolyze IAA-Ala in vivo, because the iar3 mutant is less sensitive to root inhibition by this conjugate (Fig. 2). The fact that the overlapping function of ILL2 does not completely mask the iar3 mutant phenotype suggests that the expression patterns of these two genes may differ.

IIL1 and ILL2 are adjacent genes on chromosome 5 that encode proteins that are 87% identical to one another (6, 8). In contrast to GST-ILL2, kinetic analysis of GST-IIL1 (Fig. 3 and...

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Arabidopsis Auxin Conjugate Hydrolases

TABLE I
pH dependence of Arabidopsis amidohydrolases

<table>
<thead>
<tr>
<th>Buffer (pH)</th>
<th>GST-ILR1</th>
<th>GST-IAR3</th>
<th>GST-ILL1</th>
<th>GST-ILL2</th>
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<tr>
<td>HEPES (6.5)</td>
<td>4.4</td>
<td>6.9</td>
<td>2.0</td>
<td>290</td>
<td>&lt; 1.0</td>
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<tr>
<td>HEPES (7.0)</td>
<td>10</td>
<td>26</td>
<td>5.6</td>
<td>660</td>
<td>&lt; 1.0</td>
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<tr>
<td>HEPES (7.5)</td>
<td>13</td>
<td>38</td>
<td>7.9</td>
<td>600</td>
<td>&lt; 1.0</td>
</tr>
<tr>
<td>Tris (7.5)</td>
<td>14</td>
<td>63</td>
<td>20</td>
<td>1,900</td>
<td>&lt; 1.0</td>
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<tr>
<td>Tris (8.0)</td>
<td>&lt;1.0*</td>
<td>75</td>
<td>14</td>
<td>2,200</td>
<td>&lt; 1.0</td>
</tr>
<tr>
<td>Tris (8.5)</td>
<td>&lt;1.0</td>
<td>19</td>
<td>1.8</td>
<td>1,200</td>
<td>&lt; 1.0</td>
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* The detection limit was 1.0 nmol/min/mg.

TABLE II
Cofactor dependence of Arabidopsis amidohydrolases

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<tr>
<th>Cofactor</th>
<th>GST-ILR1</th>
<th>GST-IAR3</th>
<th>GST-ILL1</th>
<th>GST-ILL2</th>
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<tr>
<td>none</td>
<td>24 ± 17</td>
<td>&lt; 5b</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
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<tr>
<td>EDTA</td>
<td>12 ± 10</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
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<tr>
<td>Mn2+</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Zn2+</td>
<td>8 ± 12</td>
<td>8 ± 7</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
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<tr>
<td>Cu2+</td>
<td>100 ± 68</td>
<td>22 ± 15</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
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<tr>
<td>Mg2+</td>
<td>28 ± 20</td>
<td>12 ± 5</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
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* Mean ± S.D. of at least three trials.

TABLE III
Substrate specificity of Arabidopsis amidohydrolases

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>GST-ILR1</th>
<th>GST-IAR3</th>
<th>GST-ILL1</th>
<th>GST-ILL2</th>
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<tbody>
<tr>
<td>IAA-Ala</td>
<td>21 ± 2</td>
<td>23 ± 5</td>
<td>15 ± 6</td>
<td>1,600 ± 130</td>
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<tr>
<td>IAA-Asn</td>
<td>&lt; 1b</td>
<td>2.9 ± 1.5</td>
<td>14 ± 3</td>
<td>25 ± 15</td>
</tr>
<tr>
<td>IAA-Asp</td>
<td>13 ± 10</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>6.5 ± 2.5</td>
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<tr>
<td>IAA-Cys</td>
<td>&lt; 1</td>
<td>2.1 ± 1.8</td>
<td>&lt; 1</td>
<td>8.1 ± 0.7</td>
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<tr>
<td>IAA-Gln</td>
<td>61 ± 7</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>56 ± 17</td>
</tr>
<tr>
<td>IAA-Glu</td>
<td>21 ± 16</td>
<td>2.5 ± 1.4</td>
<td>&lt; 1</td>
<td>14 ± 6</td>
</tr>
<tr>
<td>IAA-Gly</td>
<td>9 ± 1</td>
<td>5.3 ± 1.0</td>
<td>&lt; 1</td>
<td>46 ± 6</td>
</tr>
<tr>
<td>IAA-His</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>72 ± 15</td>
</tr>
<tr>
<td>IAA-Il</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>6.7 ± 6.6</td>
</tr>
<tr>
<td>IAA-Leu</td>
<td>56 ± 8</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>120 ± 76</td>
</tr>
<tr>
<td>IAA-Lys</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>IAA-Met</td>
<td>47 ± 9</td>
<td>7.1 ± 1.7</td>
<td>&lt; 1</td>
<td>330 ± 250</td>
</tr>
<tr>
<td>IAA-Phe</td>
<td>180 ± 23</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>170 ± 120</td>
</tr>
<tr>
<td>IAA-Pro</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>IAA-Ser</td>
<td>&lt; 1</td>
<td>4.1 ± 1.5</td>
<td>&lt; 1</td>
<td>530 ± 93</td>
</tr>
<tr>
<td>IAA-Thr</td>
<td>4 ± 3</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>320 ± 28</td>
</tr>
<tr>
<td>IAA-Trp</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>3.6 ± 0.9</td>
</tr>
<tr>
<td>IAA-Tyr</td>
<td>95 ± 14</td>
<td>16 ± 4</td>
<td>&lt; 1</td>
<td>150 ± 24</td>
</tr>
<tr>
<td>IAA-Val</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>96 ± 17</td>
</tr>
</tbody>
</table>

* Values shown are the means of at least 3 time points ± S.D.

Table IV suggests that ILL1 is unlikely to contribute to in vivo IAA conjugate hydrolysis. These results, along with the phylogenetic analysis (Fig. 1), suggest that ILL1 and ILL2 result from a recent duplication and that ILL1 may no longer be relevant.

GST-IAR3 hydrolyzes IAA-amino acid conjugates with small side chains (Table III), and preliminary experiments suggest that GST-IAR3 may also hydrolyze amino acid conjugates of the plant defense hormone jasmonic acid3. As IAR3 transcripts accumulate in response to methyl jasmonate and wounding (38), IAR3 may provide a link between wound responses and auxin homeostasis.

Although the IAA conjugates present in Arabidopsis seeds and mature plants have not been determined, recent reports indicate that the only IAA-amino acid conjugates present in Arabidopsis seedlings are IAA-Ala, IAA-Leu, IAA-Asp, and IAA-Glu (22, 23). Only GST-ILR1 and GST-ILL2 cleave IAA-Asp to a very slight degree (Table III), and 40 μM IAA-Asp is inactive in root inhibition bioassays (Fig. 2), further supporting previous data indicating that IAA-Asp is a catabolite of IAA rather than a storage form (3, 39). GST-ILR1 and GST-ILL2 can also cleave IAA-Glu, which may account for the slight root elongation inhibition induced by this conjugate. GST-IAR3 and GST-ILL2 both prefer IAA-Ala above other conjugates tested (Table III), and the high bioactivity of this conjugate (Fig. 2) supports a role for IAA-Ala in IAA storage. GST-ILR1 has the highest catalytic efficiency on IAA-Leu (Table IV), and GST-ILR1 is unique in that it hydrolyzes several bulky hydrophobic conjugates (including IAA-Phe, IAA-Tyr, and IAA-Leu) more efficiently than IAA-Ala. Because it has recently been reported that IAA-Ala accumulates in aerial tissues and IAA-Leu accu-

mulates in roots (23), it will be important to determine the expression patterns of the genes encoding these hydrolases in *Arabidopsis*. It will also be interesting to determine whether any of these hydrolases cleave the recently identified IAA-peptides from bean or *Arabidopsis* (21).

The *Arabidopsis* amidohydrolases resemble a class of microbial amidohydrolases (Fig. 1) that includes hippuricases (29) and aminoacylases that hydrolyze acetylated amino acids (27). Interestingly, an IAA-Asp hydrolase (*IaaspH*) purified from *Enterobacter agglomerans* (26, 40) is ~20% identical to the *Arabidopsis* amidohydrolases characterized here (Fig. 1). In contrast to the *Arabidopsis* enzymes, the *Enterobacter* enzyme is very specific for IAA-Asp and is inactive on other tested IAA-amino acids (40). The high *Km* (13.5 mM) of the microbial enzyme (40) suggests that the in vivo substrates of this enzyme remain to be identified, but it has been suggested that *IaaspH* might provide a useful tool for altering auxin homeostasis in transgenic plants (26, 40). In contrast to the *Arabidopsis* amidohydrolases, which do not efficiently hydrolyze IAA-Asp (Table III), heterologous expression of *IaaspH* in plants might effectively short-circuit IAA inactivation.

Genome sequencing projects are uncovering amidohydrolase homologs in plants other than *Arabidopsis*, including both monocots and other dicots. Phylogenetic analysis reveals that ILR1/ILL3, IAR3/ILL5/ILL1/ILL2, and ILL6 represent three distinct branches on this tree, suggesting that a diversity of amidohydrolase function is general to higher plants. The putative hydrolases from other plants are often more similar to a particular *Arabidopsis* amidohydrolase than the *Arabidopsis* enzymes are to each other (Fig. 1B). For example, the closest homolog of ILR1 in the data base is encoded by a partially sequenced wheat cDNA (Genbank™ accession number BE471246). This indicates that members of the amidohydrolase gene family diverged before the split between monocots and dicots. In addition, the presence of conserved homologs of *ILL6* in Medicago and tomato (Fig. 1B) makes it unlikely that *ILL6* is a pseudogene in *Arabidopsis*. It will be interesting to identify the substrates and biological roles of the enzymes in the ILL3 and ILL6 branches of the family.

It is becoming increasingly apparent that plants may use conjugates to dictate the tissue and subcellular localization of the attached IAA. Localization and activity of IAA conjugate amidohydrolases may be one of many regulatory points in the web of auxin homeostasis.

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REFERENCES


**Table IV**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th><em>Km</em> μM</th>
<th><em>Vmax</em> nmol/min/mg</th>
<th><em>kcat</em> s⁻¹</th>
<th><em>Kcat/Km</em> M⁻¹ s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST-ILR1</td>
<td>IAA-Ala</td>
<td>14 ± 6</td>
<td>14 ± 1</td>
<td>0.017</td>
<td>1,200</td>
</tr>
<tr>
<td>GST-IAR3</td>
<td>IAA-Leu</td>
<td>15 ± 5</td>
<td>58 ± 3</td>
<td>0.070</td>
<td>4,700</td>
</tr>
<tr>
<td>GST-ILL1</td>
<td>IAA-Ala</td>
<td>90 ± 21</td>
<td>20 ± 1</td>
<td>0.024</td>
<td>270</td>
</tr>
<tr>
<td>GST-ILL1</td>
<td>IAA-Leu</td>
<td>&gt; 455</td>
<td>&lt; 0.1</td>
<td>&lt; 0.00012</td>
<td>-1</td>
</tr>
<tr>
<td>GST-ILL2</td>
<td>IAA-Ala</td>
<td>&gt; 413</td>
<td>&gt; 31</td>
<td>&gt; 0.037</td>
<td>~2</td>
</tr>
<tr>
<td>GST-ILL2</td>
<td>IAA-Leu</td>
<td>&gt; 413</td>
<td>&gt; 0.8</td>
<td>&gt; 0.001</td>
<td>~2</td>
</tr>
<tr>
<td>GST-ILL1</td>
<td>126 ± 30</td>
<td>220 ± 29</td>
<td>2.1</td>
<td>40,000</td>
<td>1,200</td>
</tr>
</tbody>
</table>

*a* GST-ILR1 (72.5 kDa) was assayed at a concentration of 5 ng/μl.

*b* GST-IAR3 (72.9 kDa) was assayed at a concentration of 5 ng/μl.

*c* GST-ILL1 (72.6 kDa) was assayed at a concentration of 10 ng/μl.

*d* GST-ILL2 (72.6 kDa) was assayed at a concentration of 1 ng/μl.

*e* The detection limit was 0.1 nmol/min/mg.
20452

Arabidopsis Auxin Conjugate Hydrolases

Characterization of a Family of IAA-Amino Acid Conjugate Hydrolases from Arabidopsis
Sherry LeClere, Rosie Tellez, Rebekah A. Rampey, Seiichi P. T. Matsuda and Bonnie Bartel

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