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Jasmonate Response Locus *JAR1* and Several Related Arabidopsis Genes Encode Enzymes of the Firefly Luciferase Superfamily That Show Activity on Jasmonic, Salicylic, and Indole-3-Acetic Acids in an Assay for Adenylation

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Jasmonic acid (JA) and related cyclopentanones are critical plant signaling molecules, but their mode of action at the molecular level is unclear. A map-based approach was used to identify the defective gene in the Arabidopsis JA response mutant *jar1-1*. *JAR1* is 1 of 19 closely related Arabidopsis genes that are similar to the auxin-induced soybean *GH3* gene. Analysis of fold predictions for this protein family suggested that JAR1 might belong to the acyl adenylate-forming firefly luciferase superfamily. These enzymes activate the carboxyl groups of a variety of substrates for their subsequent biochemical modification. An ATP-PPi isotope exchange assay was used to demonstrate adenylation activity in a glutathione *S*-transferase–JAR1 fusion protein. Activity was specific for JA, suggesting that covalent modification of JA is important for its function. Six other Arabidopsis genes were specifically active on indole-3-acetic acid (IAA), and one was active on both IAA and salicylic acid. These findings suggest that the *JAR1* gene family is involved in multiple important plant signaling pathways.

INTRODUCTION

The jasmonate family of plant signal molecules is involved in a variety of critical functions, including defense against pathogens and insects (Farmer and Ryan, 1990; Penninckx et al., 1996; McConn et al., 1997), protection from ozone damage (Overmyer et al., 2000; Rao et al., 2000), and reproductive development (McConn and Browse, 1996; Sanders et al., 2000; Stintzi and Browse, 2000). Despite their importance, our understanding of how jasmonates work at the biochemical level still is limited.

An understanding of jasmonate signaling is complicated by the presence of multiple cyclopentanones that are biologically active in plants. Jasmonic acid (JA) is considered the primary signal in protective alkaloid production in *Eschscholtzia californica* cell cultures (Haider et al., 2000), and JA is required for male fertility in Arabidopsis (Sanders et al., 2000; Stintzi and Browse, 2000). Overexpression of a JA methyltransferase gene increases resistance to *Botrytus cinerea*, suggesting that JA methylester (MeJA) is either a primary signal or at least positively influences this pathogen defense response (Seo et al., 2001). MeJA also rescues a defect in pathogen defense response in an Arabidopsis mutant that does not synthesize linolenic acid–derived cyclopentanones (Vijayan et al., 1998).

On the other hand, the JA biosynthetic precursor 12-oxophytodienoic acid (OPDA) also is an effective signal and in some cases induces responses that are distinct from those induced by JA (Weiler et al., 1993; Mueller, 1997; Weber et al., 1997; Gundlach and Zenk, 1998). Recent evidence demonstrated that JA and MeJA are not required for all jasmonate responses in Arabidopsis, because insect and fungal resistance were maintained in *opr3*, a mutation that blocks JA biosynthesis beyond OPDA (Stintzi et al., 2001). Analysis of *opr3* also suggested that JA/MeJA regulates OPDA levels. Other jasmonates that potentially influence jasmonate signaling include additional intermediates of JA biosynthesis, JA–amino acid and JA-glucosyl conjugates (Sembdner and Parthier, 1993), and dinor OPDA, which is synthesized from hexadecatrienoic acid rather than linolenic acid (Weber et al., 1997). Together, the evidence on jasmonate signaling suggests a complex interaction between jasmonate family members that may allow plants to orchestrate specific responses to a variety of stimuli (Reymond et al., 2000).

Mutations at three Arabidopsis loci (*JAR1*, *COI1*, and *JIN1*) reduce sensitivity to JA, suggesting that they might affect jasmonate signal transduction (Staswick et al., 1992; Feys et al., 1994; Berger et al., 1995). Consistent with this

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idea, *COI1* encodes an F-box protein that is related to TIR1, a component of a ubiquitin-like E3 complex that is involved in plant auxin response (Ruegger et al., 1998; Xie et al., 1998). Although other jasmonate signaling components have not been characterized, analysis of *jar1-1* has shown that this locus is involved in protection against a variety of stresses that plants encounter. These include resistance to the opportunistic soil fungus *Pythium irregulare* (Staswick et al., 1998), systemic resistance pathways that protect against various other pathogens (van Loon et al., 1998; Clarke et al., 2000) and limiting damage from ozone exposure (Overmyer et al., 2000; Rao et al., 2000). On the other hand, *jar1-1* plants are fertile even though JA is required for male fertility (Sanders et al., 2000; Stintzi and Browse, 2000), suggesting that *JAR1* is not required for all jasmonate responses.

To better understand its function at the molecular level, we identified the *JAR1* gene using a molecular mapping approach and then determined the function of the JAR1 protein by fold prediction modeling and an in vitro biochemical assay. Surprisingly, rather than acting as a signaling intermediate, the role of JAR1 apparently is to biochemically modify JA itself.

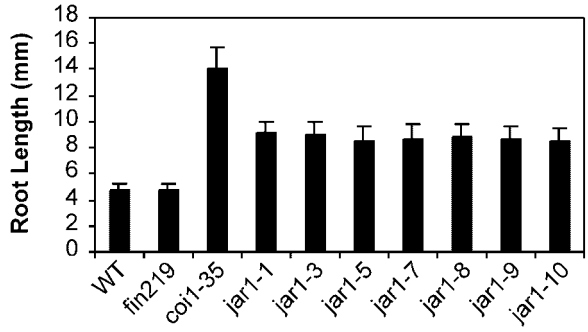

Figure 1. Comparison of the Sensitivity of Arabidopsis Mutants to MeJA.

Seedlings were grown for 5 days on vertical agar plates containing 50 μM MeJA. Bars indicate mean root length of 19 seedlings with standard errors. WT, wild type.

RESULTS

jar1-1 Is Representative of Other *jar1* Alleles

jar1-1 exhibits moderate insensitivity to MeJA in root growth inhibition assays and normal male fertility. This is in contrast with the male sterility and stronger JA insensitivity of *coi1* (Figure 1). To establish whether *jar1-1* is merely a weak allele of this locus, several new alleles were isolated and characterized. A screen for MeJA resistance among $\sim 2 \times 10^5$ M2 seedlings representing 5×10^4 ethyl methanesulfonate–mutagenized M1 parents yielded six new *jar1* alleles, based on their inability to complement the original *jar1-1* mutation. All new *jar1* alleles were indistinguishable from *jar1-1*, showing normal male fertility and the same degree of root growth in the presence of MeJA (Figure 1). These findings suggest that phenotypes reported previously for *jar1-1* represent the full effect of a lesion at this locus and that *JAR1* is not necessary for male fertility.

JAR1 Belongs to a Multigene Family That Includes Soybean *GH3*

JAR1 was mapped previously to the right end of chromosome II between phenotypic markers *AS1* and *CER8* (Staswick et al., 1998). Cleaved amplified polymorphic sequence and simple sequence length polymorphism markers were used to refine the location to within an 80-kb interval flanked by molecular markers F11C10K/L and F13A10C/D (Figure 2). Wild-type DNA segments from this region were subcloned from BAC clones F11C10 and F13A10 into the plant transformation vector pPZP212 and then introduced into *jar1-1* plants to test for complementation. Progeny of the primary transformants were assayed for restored sensitivity to MeJA. Only the 15.1-kb PstI and the 5.1-kb BamHI fragments from BAC F11C10 complemented the defect in *jar1-1*, segregating ~3 sensitive:1 insensitive to MeJA. The only complete open reading frame (ORF) on BamHI 5.1 is F11C10.6, which also is present on PstI 15.1 but not on the noncomplementing fragments. F11C10.6 is predicted to encode 576 amino acids and a protein of 64,350 D (Figure 3).

Searches of the complete Arabidopsis genome revealed that F11C10.6 is 1 of 19 closely related genes that are distributed on four of the five chromosomes (Figure 4). These genes are predicted to encode proteins having 47 to 91% sequence identities with each other. The homology extends throughout the entire protein coding regions, suggesting that they have a similar biochemical function (Figure 3). Related genes occur in several other plants as well, including the well-characterized auxin-induced *GH3* gene from soybean (Hagen et al., 1991) and an auxin-inducible tobacco homolog (Roux and Perrot-Rechenmann, 1997). However, the biochemical functions of these genes are unknown. Also of unknown function are ORFs of similar size from human and mouse, which have lower sequence similarity to the plant genes.

cDNA corresponding to the F11C10.6 ORF from *jar1-1* was sequenced to identify a possible mutation responsible for the gene defect. The only variance from the predicted wild-type ORF was the substitution of Phe for Ser-101. Ser is found in 17 of the 19 Arabidopsis genes at this position, and the two exceptions (T26J12.7 and T22N19.30) have a conservative substitution of Thr. Sequence analysis of the six new *jar1* alleles revealed that they also contained mutations consistent with a defective gene (Figure 3). Nucleotide

substitutions in *jar1-7* and *jar1-9* introduce premature stop codons, whereas a deletion in *jar1-8* shifts the reading frame. Functionally informative is *jar1-3*, which substitutes a Lys for Glu-334, which is invariant in the Arabidopsis proteins. The Arg substituted for Gly-303 in *jar1-5* does not alter a conserved residue, although none of the Arabidopsis proteins predict a charged amino acid at this position. This suggests a possible effect on protein structure. Mutant *jar1-10* was from a T-DNA–mutagenized population and contained a disrupted reading frame as a result of a 16-bp deletion. Together, these results confirm that ORF F11C10.6 represents the locus responsible for JA/MeJA insensitivity in *jar1* alleles.

JAR1 Is Structurally Related to the Firefly Luciferase Superfamily of Adenylate-Forming Enzymes

Homology searches with the amino acid sequence of JAR1 did not yield a match with a protein of known biochemical function. Similarly, searches with possible motifs built from highly conserved regions of the Arabidopsis JAR1-like sequences did not match currently recognized protein motifs in the databases. Therefore, we initiated a structural analysis to search for a protein fold class to which JAR1 and its homologs might belong. Each was analyzed with a fold recognition algorithm using the 3D-PSSM World Wide Web–based server (Kelley et al., 2000). Although none of the Arabidopsis proteins returned a high probability for a match with a known structure, among the 20 candidate matches for JAR1 was firefly luciferase. Despite their low overall primary sequence identity (13%) and weak probability of a match (expectation value [E] = 3.32), firefly luciferase was the only candidate structure of comparable size with JAR1 (>500 amino acids).

Analysis of the model for JAR1 showed that the regions of predicted fold similarity extended throughout the entire luciferase and JAR1 proteins, although regions of predicted structural similarity were interrupted by numerous sequence deletions and insertions in JAR1. The JAR1-related mouse protein D11lgp1 also was analyzed in a similar manner. It yielded a robust 3D-PSSM E value of 2.9×10^{-4}, for a fold similarity with the Phe-activating domain of gramicidin synthetase 1 (GrsA) from *Bacillus brevis*. This E value represents a high degree of confidence in the structural relatedness of GrsA and D11lgp1 (Kelley et al., 2000).

GrsA and firefly luciferase belong to the firefly luciferase–like structural superfamily. These proteins share a four-domain structure consisting of duplicated α/β folds, a β-barrel, and an $\alpha+\beta$ domain at the C terminus. GrsA and firefly luciferase are the only family members whose crystal structures are solved, but many other members have been identified based on functional and structural similarities (Conti et al., 1997). Firefly luciferase–like family members typically have low sequence identity but nevertheless are functionally and structurally related (Kleinkauf and Von Dohren, 1996). The common biochemical reaction they perform on a wide

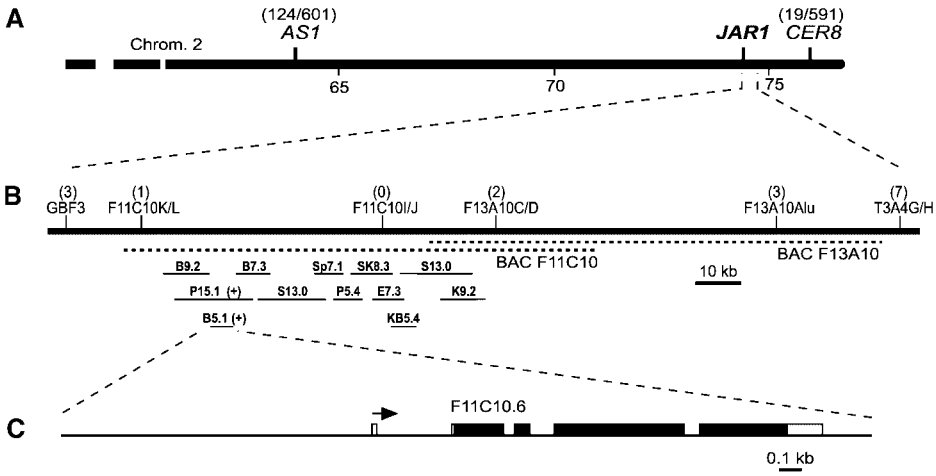

Figure 2. Positional Cloning of *JAR1*.

(A) The location of *JAR1* on the right end of chromosome II is shown relative to phenotypic markers *AS1* and *CER8*. Fractions indicate number of recombinant chromosomes between *JAR1* and the respective markers.

(B) Molecular mapping of *JAR1*. Numbers in parentheses above markers indicate number of recombinant chromosomes between *JAR1* and the respective markers. Dashed lines indicate BAC clones, and short lines with labels depict subclones used to test for complementation of *jar1-1*. Restriction endonucleases used to generate subclones (B, BamHI; E, EcoRI; K, KpnI; P, Pst1; S, SacI; Sp, SpeI) and their sizes (in kb) are shown. Complementing subclones are indicated by (+).

(C) Structure of the single ORF on complementing subclone B5.1. Black and white bars represent predicted coding and noncoding transcribed regions, respectively, and intervals between bars represent introns (www.tigr.org/tdb/ath1/htmls/index.html).

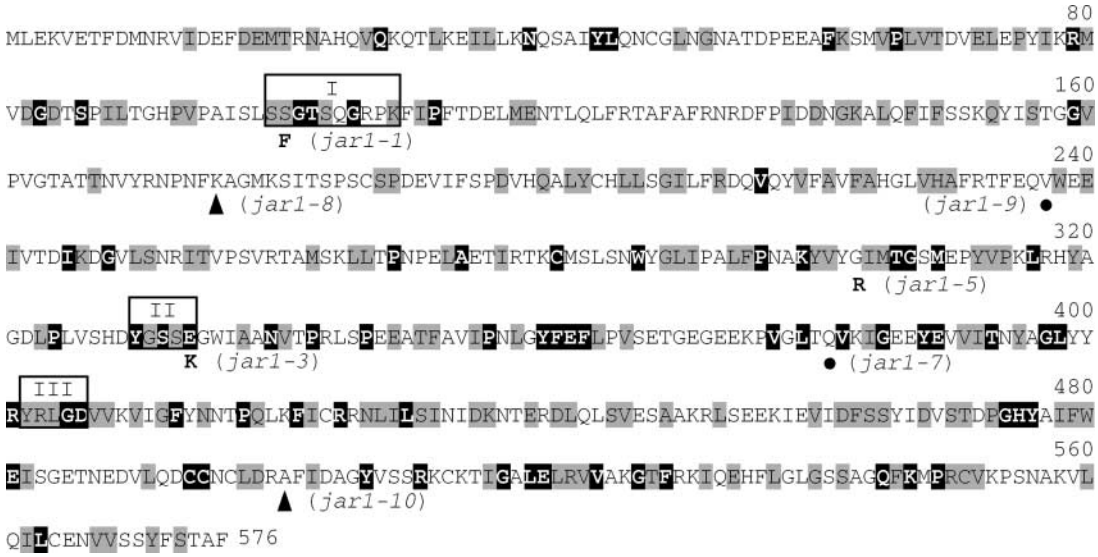

Figure 3. The JAR1 Sequence and Its Similarity to 18 Closely Related Arabidopsis Proteins.

The positions of mutations in various *jar1* alleles are indicated below the sequence by single letters (amino acid substitutions), dots (premature stop codons), or triangles (deletions). Residues highlighted in black are invariant, and those highlighted in gray are similar among at least 16 of the 19 Arabidopsis proteins. Gaps introduced by PileUp (Genetics Computer Group, Madison, WI) to optimize alignment are not shown. Open boxes identify conserved motifs in the firefly luciferase–like adenylate-forming enzyme superfamily.

variety of substrates is the MgATP-dependent activation of carboxyl groups with AMP. Activated substrates then undergo a variety of biochemical reactions, including peptide and polyketide synthesis (e.g., nonribosomal peptidyl synthetases), acyl:CoA formation involving fatty acids and organic acids (e.g., acyl:CoA ligases/synthetases), and oxidation of adenylated luciferin (luciferase).

A hallmark of many, although not all, adenylate-forming domains of this superfamily is the presence of three short and weakly conserved sequence motifs that are involved in ATP/AMP binding (Chang et al., 1997). All three motifs are found in the plant JAR1-like proteins, and many of the motif residues are invariant in the Arabidopsis proteins (Figure 3). The three motifs in JAR1 and its homologs are in a similar relative position as in other acyl adenylate-forming enzymes, except that motif 1 is displaced ~100 residues upstream. The mutations in *jar1-1* and *jar1-3* substitute highly conserved residues in motifs 1 and 2, respectively, supporting the functional relevance of these motifs in JAR1. Other functional residues of adenylate-forming enzymes that are conserved in JAR1 include Gly-307 and Arg-424, both of which are invariant in the Arabidopsis JAR1 homologs.

JAR1 Is Specifically Active on JA in an Assay for Adenylation

The standard assay for adenylate-forming enzyme activity is substrate-dependent ATP-^{32}P-PPi isotope exchange (substrate + ATP ↔ substrate-AMP + PPi) (Pavela-Vrancic et al., 1994). We constructed a glutathione *S*-transferase (GST)–JAR1 fusion protein encompassing the complete JAR1 coding region and expressed it in *Escherichia coli*. Glutathione-agarose–purified GST-JAR1 yielded a protein of the expected size, ~95 kD (Figure 5A). The agarose-bound fusion protein was tested against a variety of carboxyl-containing substrates for their ability to support isotope exchange. Only JA yielded exchange of ^{32}P-PPi into ATP (Figure 5B). Controls demonstrated the need for JA, Mg, ATP, and JAR1 enzyme (Figure 5C), and the requirement for a free carboxyl group was shown by the inactivity of JAR1 on MeJA. A high level of substrate specificity also was evident from the inactivity of JAR1 on the JA biosynthetic precursors OPDA and linolenic acid as well as the other plant carboxylic acids tested.

Several JAR1 Family Members Are Active on Auxin

We next investigated whether JAR1 homologs also were active on JA or whether they showed activity with other substrates. The substrate specificity of adenylate-forming enzymes is determined partially by residues between motifs 1 and 2 that form a substrate binding pocket (Conti et al., 1997). Because this region is the least conserved among the Arabidopsis proteins (Figure 3), we suspected that at least some of them might vary in their substrate specificity. Therefore, in addition to JA, they were assayed with other

important carboxyl-containing plant signaling molecules. GST fusions were made for each of 14 other genes and assayed in the same manner described above. No other fusion protein exhibited activity on JA. However, seven were active on indole-3-acetic acid (IAA). Representative examples of the substrate specificity are shown in Figure 5D, and the results for all proteins that were assayed are summarized in Figure 6. Interestingly, M4I22.70 showed activity on both IAA and the pathogen defense signal salicylic acid (SA). T26I20.12 was not obtained by reverse transcriptase–mediated PCR, so it could not be examined here. The remainder of the fusion proteins tested did not exhibit activity with any of the substrates investigated.

The JAR1-like proteins cluster into three sequence homology groups that are consistent with their substrate specificities (Figure 6). Group I has sequence features that are distinct from those of the others and includes only two members, JAR1 and F9H3.1. The substrate specificity of the latter is unknown at present. At least seven of the eight group II proteins apparently are involved in IAA adenylation, whereas group III appears to be active on substrates other than the plant hormones tested. The group III proteins that were not tested are ~90% identical in sequence to T22N19.20, so they most likely would have substrate specificities similar to those of that protein.

JAR1 Is Not Required for Far-Red Light Response

JAR1 mapped to the same locus as that identified recently in mutant *fin219*, a suppressor of *cop1* (Hsieh et al., 2000). Because *fin219* exhibited increased hypocotyl elongation in far-red light, we examined the *jar1* alleles for their far-red light response. We found increased hypocotyl elongation in *fin219* and *phyA* as expected, but no difference from the wild type was detected in any of our *jar1* alleles (Figure 7). We also examined *fin219* for altered response to MeJA. Unlike the *jar1* alleles, *fin219* showed no increase in resistance to MeJA (Figure 1). Because several of our *jar1* alleles are likely to be functionally null because of premature protein termination, we conclude that the loss of JAR1 activity does not lead to detectable defects in far-red light response in hypocotyl elongation.

DISCUSSION

The discovery that JAR1 is biochemically active on JA was surprising, because we had anticipated that the protein associated with this response locus might be a signal transduction intermediate. Rather, it appears that JAR1 modifies at least one of the signal molecules involved in jasmonate response. Firefly luciferase–like adenylate-forming enzymes occur in both prokaryotes and eukaryotes, in which they activate carboxyl groups on a wide variety of substrates for their further biochemical modification. Adenylation by some of these enzymes leads to regulatory control of important cellular processes (Black et al., 2000). The results presented here suggest that adenylation also is involved in the control of plant hormone activity.

JAR1 Represents a New Class of Adenylate-Forming Enzymes

Members of the firefly luciferase superfamily characteristically have low sequence identity despite their related biochemical function in a variety of organisms. Other members of this superfamily occur in plants, including 4-coumarate:CoA ligases (4CL), which activate 4-coumarate in the last step of phenylpropanoid metabolism (Stuible et al., 2000). However, plant 4CLs share no significant sequence identity with JAR1-like proteins, except for limited similarity in motif 1. Motifs 2 and 3 are not found in plant 4CLs, and

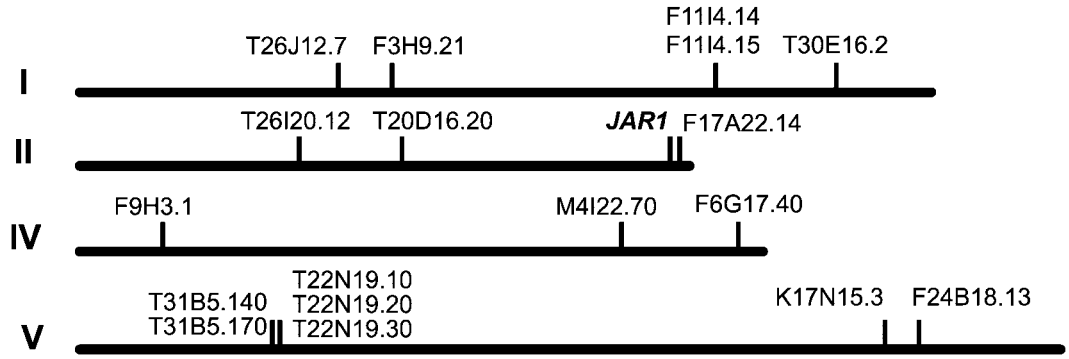

Figure 4. Chromosomal Distribution of the Arabidopsis *JAR1*-Like Gene Family.

Genes are designated by the BAC open reading frame numbers assigned by the Arabidopsis Genome Initiative (www.arabidopsis.org). Chromosome numbers are shown at left. No homologs exist on chromosome III.

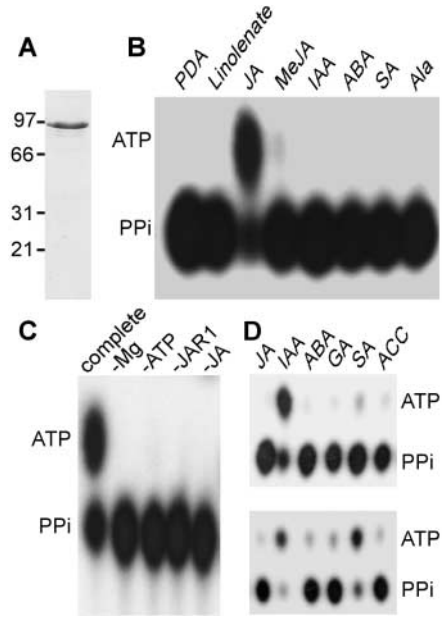

Figure 5. Biochemical Assay for JAR1 and Related Enzyme Activities.

(A) Analysis of purified GST-JAR1 fusion protein on a Coomassie blue–stained polyacrylamide gel. Positions of molecular mass markers (kD) are shown at left.

(B) Substrate specificity of JAR1 determined by ATP-^{32}P-PPi exchange. The positions of PPi and ATP after chromatography are indicated. Lanes are labeled with the substrates assayed.

(C) The first lane contains the complete reaction mixture with JA as in **(B)**. Other lanes are the same as the first minus the component indicated.

(D) Representative example of a substrate specificity assay for proteins related to JAR1. The top chromatograph shows T20D16.20; the bottom chromatograph shows M4I22.70. Reaction conditions are the same as those described above.

ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; GA, gibberellic acid; PDA, 12-oxophytodienoic acid.

4CLs contain an absolutely conserved box II motif that is not evident in the JAR1 family. These facts demonstrate the usefulness of structural modeling to identify protein function when direct sequence comparisons are not fruitful.

Based on their high level of sequence homology and their lack of similarity with previously recognized family members, JAR1-like family members represent a new class of acyl adenylate-forming enzymes. These are found mostly in plants, although lower levels of sequence similarity are evident in proteins predicted to occur in mouse, human, and *Clostridium acetobutylicum*. Besides the 19 Arabidopsis genes and the previously characterized soybean *GH3* (Hagen et al., 1991), other genes closely related to *JAR1* occur in a variety of monocotyledonous and dicotyledonous plants. At present, these are represented mostly as ESTs in the sequence databases. The moss *Physcomitrella patens* also apparently contains a gene that encodes a protein with ~42% identity with JAR1. We suggest that all plant species are likely to have JAR1-like acyl adenylate-forming enzymes, and some of these undoubtedly are active in hormone function.

Biochemical Roles for the Adenylation of JA, SA, and IAA

The fate of substrates adenylated by other firefly luciferase–like enzymes suggests several possible biochemical roles for the activation of plant hormone carboxyl groups. First, the adenylation of amino acids and other substrates can initiate the synthesis of a diverse array of peptide and polyketide secondary metabolites (Kleinkauf and Von Dohren, 1996). In these cases, acyl adenylates serve as donors for the formation of thioester intermediates with either CoA or an enzyme-linked pantetheine, which then donates the acyl group to an amine or alcohol (Chang et al., 1997). Adenylation of JA, SA, and IAA might initiate their conjugation to amino acids or other adducts, thereby regulating hormone activity. Amino acid and glucosyl conjugates of JA, IAA, and other hormones are well documented in plants, and IAA conjugates play an important role in regulating the availability of free auxin, which is the biologically active form (Sembdner and Parthier, 1993; Normanly, 1997; Tam et al., 2000). Although the biosynthetic pathway for IAA glucosyl conjugation in maize is known (Szerszen et al., 1994), the mechanism of amino acid conjugation to plant hormones has not been determined.

A second possible function for adenylation is in the control of hormone cellular location. Fatty acyl:CoA ligases can control directional membrane transport of fatty acids, thereby regulating their bioactivity (Black et al., 2000). *JAR1* and related genes might play a similar regulatory role for plant hormones. A third possibility is that adenylation of JA controls its turnover, analogous to the manner in which CoA thioesters of animal fatty acids and eicosanoids are degraded in peroxisomes (Mannaerts et al., 2000). Other reactions initiated by adenylation in this enzyme family include the oxidation of luciferin (Conti et al., 1997) and the reduction of α-aminoadipate (Ehmann et al., 1999), but it is not clear how these might relate to the control of hormone response in plants.

The Role of JAR1 in Jasmonate Signaling

An understanding of how adenylate formation might influence jasmonate response is complicated by our incomplete knowledge of how jasmonate signaling is coordinated through different cyclopentanones. As discussed above, there is evidence that OPDA and JA/MeJA are each sufficient for signaling at least some Arabidopsis defense responses, and JA/MeJA may regulate the endogenous level

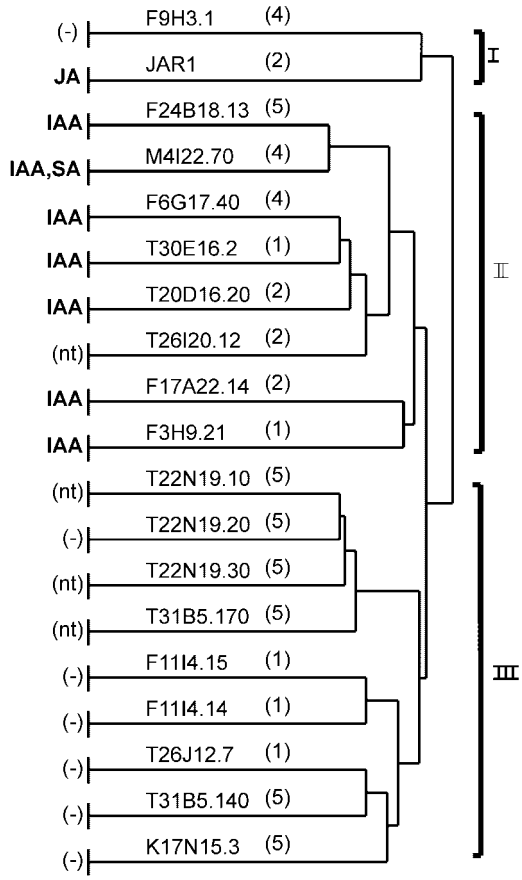

Figure 6. Summary of Sequence Homology and Substrate Specificity Relationships among Arabidopsis JAR1-Like Proteins.

Pair-wise similarity tree constructed with PileUp (Genetics Computer Group) using the blosum 62 scoring matrix. Numbers in parentheses indicate chromosomes. Substrate specificity for each protein and sequence homology subgroups are indicated at left and right, respectively. Four genes not tested (nt) were not obtained by reverse transcriptase–mediated PCR or were nearly identical to others. Inactivity on substrates tested (see Fig. 5) is indicated by (−).

of OPDA (Vijayan et al., 1998; Seo et al., 2001; Stintzi et al., 2001). JA/MeJA, OPDA, and other cyclopentanones appear to work together in a complex signaling network.

Of the 15 proteins we assayed, only JAR1 exhibited biochemical activity with JA. Although F9H3.1 is grouped with JAR1 on the basis of sequence homology, it is only slightly more related to JAR1 than are other family members, and it was not active with JA, OPDA (data not shown), or any other plant hormone as substrate. We verified by sequence analysis that mutations were not introduced inadvertently into this gene. Based on sequence homologies with the other proteins (Figure 6), it is likely that the four untested proteins also are inactive on JA. Thus, only one gene appears to be associated with JA adenylation. This finding suggests that adenylation is not required for all jasmonate-signaled responses, because all *jar1* alleles were fertile despite the requirement of JA for pollen fertility (McConn and Browse, 1996).

Our findings imply that the metabolism of JA by JAR1 positively regulates jasmonate signaling, because *jar1* is defective in response. If JAR1 initiates the conjugation or degradation of JA, this would seem counterintuitive, suggesting that free JA is not biologically active. By contrast, free IAA is active, whereas conjugated forms function in storage, transport, and catabolism (Normanly, 1997). A possible explanation is that JA signaling is feedback inhibited by the overaccumulation of JA or that high levels of JA interfere with signaling by other cyclopentanones such as OPDA. The role of JAR1 could be to regulate JA levels to coordinate signaling with other jasmonates. We also cannot exclude the possibility that JAR1 is active on other cyclopentanones that influence jasmonate signaling, although our results indicate that JAR1 is inactive on OPDA.

In contrast with that of JA, the adenylation of IAA appears to negatively regulate auxin response. Overexpression of *DFL1*, the gene that encodes ORF F24B18.13, decreases auxin sensitivity and inhibits auxin-signaled growth responses (Nakazawa et al., 2001). This is consistent with a mechanism that inactivates auxin. Amino acid conjugates of IAA appear to play a key role in regulating auxin catabolism (Normanly, 1997), but whether the JAR1-like proteins are involved in IAA conjugation or in other auxin metabolic pathways remains to be determined. It is not necessarily the case that adenylation leads to the same biochemical outcome for JA and IAA.

Overexpression of a gene encoding JA methyltransferase activates jasmonate responses in transgenic Arabidopsis,

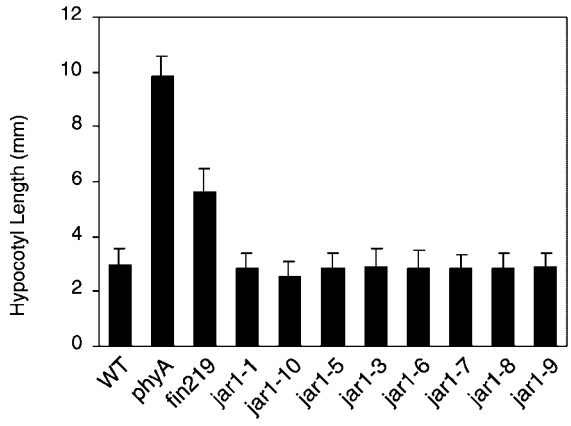

Figure 7. Response of *jar1* Alleles to Far-Red Light.

Seedlings were grown for 5 days at room temperature under far-red light. Bars indicate mean hypocotyl length of 25 seedlings with standard errors. WT, wild type.

suggesting that MeJA is biologically active (Seo et al., 2001). The *S*-adenosyl-L-Met:carboxyl methyltransferase does not require JA adenylation, indicating that JAR1 is not involved in this reaction. On the other hand, *JAR1* mutants confer the same level of resistance to both JA and MeJA when assayed for inhibition of root growth (Staswick et al., 1998). This implies that response to MeJA first requires demethylation and then adenylation, because MeJA is not a substrate for JAR1. It is not clear what accounts for this apparent discrepancy, but response to exogenous JA and MeJA in root growth assays may not accurately reflect their endogenous signaling roles. *jar1* is not affected in root development in the absence of exogenous jasmonate. This suggests that JAR1 is not involved directly in normal root growth but nevertheless influences a stress response to exogenous JA and MeJA. The same observation is true for *coi1* and for several other hormone-insensitive mutants.

It is possible that JAR1 provides an alternate pathway to JA methylation. Some acyl adenylates form thioester intermediates that in turn donate the activated acyl group to an alcohol acceptor (Chang et al., 1997). If methanol were an acceptor for JA thioester, this would yield MeJA. Two independent mechanisms for JA methylation might explain the intermediate resistance of *jar1* alleles compared with *coi1* as well as the lack of a role for JAR1 in male fertility.

Substrates activated by the adenylate-forming superfamily typically release AMP upon formation of a substrate-thioester intermediate with either CoA or with a protein-appended phosphopantetheine (Kleinkauf and Von Dohren, 1996). Adding CoA to the GST-JAR1 adenylation reaction with JA as the substrate did not release AMP (data not shown). This finding suggests that further metabolism of activated JA may require the covalent linkage of phosphopantetheine to JAR1, or to another carrier protein, by a specific phosphopantetheinyl transferase (Gehring et al., 1997). Comparison of the JAR1 sequence with those of other adenylate-forming enzymes also suggests that it does not contain catalytic domains other than the one for adenylation. Thus, biochemical steps downstream of adenylation by JAR1 likely involve other proteins. Identification of these proteins and the biochemical reactions they are involved in should help clarify the mechanism by which the modification of JA influences jasmonate signaling.

Redundancy for Enzyme Activity Associated with Auxin

We established that at least seven of the eight group II family members are active on IAA (Figure 6). *DFL1* (F24B18.13) was discovered recently by activation tagging in Arabidopsis, and its overexpression caused auxin response phenotypes (Nakazawa et al., 2001), consistent with our determination of the biochemical role of the gene product. This finding indicates that auxin adenylation also is important in plants. Redundancy for the genes involved in IAA modification may have prevented their earlier association with auxin activity, despite extensive mutant screens for defects in auxin response. It is possible that differential expression of group II family members helps coordinate diverse auxin responses throughout the plant.

One protein showing biochemical activity with IAA (M4I22.70) also was active with SA, but not with other hormones tested. Although the functional relevance of this observation awaits further study, it is intriguing. Like JA, SA is a critical component of pathogen defense responses, although JA and SA act in largely independent pathways (Penninckx et al., 1996; Bowling et al., 1997; Pieterse et al., 1998). It will be important to determine whether mutations in M4I22.70 or its overaccumulation alters defense pathways in which SA is known to function. Group III family members appear not to be active on the common plant hormones assayed here. Whether they are involved in other signaling pathways or in different metabolic processes remains to be determined. The fact that we obtained 15 clones by reverse transcriptase–mediated PCR indicates that most, if not all, members of the *JAR1*-like gene family are expressed in Arabidopsis.

Auxin and Jasmonate Response May Be Linked through *JAR1*

The similarity of *COI1* to an auxin signaling component suggests that the control of JA and auxin response follow similar pathways. JAR1 may provide another important link between JA and auxin signaling. The soybean *JAR1* homolog *GH3* is induced rapidly by low levels of auxin (Hagen et al., 1991), and limited study suggests that *JAR1* also is auxin inducible in Arabidopsis (Hsieh et al., 2000). This could provide a mechanism by which auxin influences JA response. We recently found another link between JA and IAA in that the auxin response mutant *axr1* also is defective in JA responses (I. Tiryaki and P.E. Staswick, unpublished data).

Influence of *JAR1* in Far-Red Light Response

Although other workers found that a defect in *JAR1* (*fin219*) reduced far-red light response, we did not find evidence of similar defects in our *jar1* alleles. This discrepancy is possibly explained by the fact that the mutation in *fin219* apparently is epigenetic, involving aberrant promoter methylation (Hsieh et al., 2000). Misregulation of *JAR1* expression in *fin219* might lead to an altered far-red light response, whereas the loss of JAR1 protein activity in our mutants did not. Overexpression of *JAR1* in wild-type plants also reportedly results in a weak hypersensitive response to far-red light (Hsieh et al., 2000), but it remains to be seen whether this is a direct effect of JAR1 enzymatic activity. Alternatively, misregulation or constitutive expression of *JAR1* could alter the expression of other genes involved in light re-

sponse. One possible mechanism could be the cosuppression of homologous genes. The fact that overexpression of the auxin-adenylating family member *DFL1* (F24B18.13) alters light response could support the latter explanation (Nakazawa et al., 2001).

In summary, we identified a biochemical activity previously unrecognized to be involved in plant hormone metabolism. Evidence from the *JAR1* mutants suggests that adenylation plays an important role in jasmonate signaling, and we propose that the same is true for IAA. Because similar genes occur widely in plants, these results should be broadly applicable, leading to a clearer understanding of the mechanism and signaling role of hormone modification in plants.

METHODS

Plant Material, Isolation of Mutants, and Growth Response

M2 seeds of ethyl methanesulfonate–mutagenized *Arabidopsis thaliana* (ecotype Columbia) were purchased from Lehle Seed (Round Rock, TX), and seeds from T-DNA–transformed lines originating from T. Jack (CS19651) were from the ABRC (Ohio State University, Columbus). Screening for long-rooted seedlings in the presence of 50 μM jasmonic acid methylester (MeJA) was on agar plates with surface-sterilized seeds as described previously (Staswick et al., 1992), except that Murashige and Skoog (1962) media agar was 1% at pH 6.0. Plates were incubated at 4°C for at least 4 days to promote uniform germination and then placed vertically in a culture chamber at 23°C under continuous fluorescent light.

Only roots growing within the agar bed were scored for root length. Putative resistant seedlings were transferred to soil, and seeds harvested from individual plants were tested again for MeJA resistance. Resistance was verified in the next generation, and allelism was determined with F1 seeds from crosses with *jar1-1*. Far-red light response was assayed on Murashige and Skoog (1962) agar plates without MeJA. Plates were incubated in a light-tight box (24 × 30 cm) covered with a far-red light filter (acrylic sheet, FRF700; Westlake Plastics, Lenni, PA) as described by Whitelam et al. (1993). Light was supplied with a 50-W Spot Gro incandescent bulb (Sylvania, St. Marys, PA) filtered through 2.5 cm of water to limit infrared radiation. Room air was circulated continuously through the box.

Map-Based Cloning

DNA for PCR-based mapping was obtained from F3 families derived from recessive *jar1-1* F2 plants that previously showed recombination between the *JAR1* locus and the flanking phenotypic markers *as1* and *cer8* (Staswick et al., 1998). DNA was isolated in 200 mM Tris-HCl, pH 7.4, 250 mM NaCl, 25 mM EDTA, and 0.5% (w/v) SDS as described by Yu and Pauls (1994). GBF3 is a HindIII cleaved amplified polymorphic sequence (CAPS) marker described previously (genome-www3.stanford.edu). F11C10K/L, F11C10I/J, F13A10C/D, and F13A10Alu were derived with information obtained from the Cereon Arabidopsis Polymorphism Collection for Landsberg *erecta* and Columbia (www.arabidopsis.org/cereon/index).

Primers used were F11C10K/L (42-bp deletion in Landsberg *erecta*, 5′-CAGTTTCCCCGACGTTAATTTC-3′ and 5′-TCTGTGGACCCACCAATGAG-3′), F11C10I/J (15-bp deletion in Landsberg *erecta*, 5′-TCACTCTCATACCACTGTCTG-3′ and 5′-CCGCTTTGATTCTGCCTACC-3′), F13A10C/D (5-bp deletion in Landsberg *erecta*, 5′-CAAAAGGAAAGTGGTAGGAAG-3′ and 5′-CCGTTGACAATAAACAAAGG-3′), and F13A10Alu (AluI CAPS, 5′-TGTAGACTTTTTGGTAGAGATG-3′ and 5′-CAAACTTTCTCTCAATCTCC-3′). T3A4G/H is a ScaI CAPS marker obtained from primers 5′-GCGGGAGTGGGGATCTCGA-3′ and 5′-GAAGATGTAAGGGAACAAAGCA-3′. BAC clones were obtained from the ABRC. Wild-type DNA subclones were tested for complementation by transformation into *jar1-1*. Restriction fragments from appropriate BAC clones were subcloned into the plant transformation vector pPZP212 and then introduced into plants by the floral dip method (Clough and Bent, 1998) with selection of transformed progeny on kanamycin plates.

Protein Structural Analysis

The 3D-PSSM protein fold recognition modeler (http://www.bmm.icnet.uk/~3dpssm/) was used to search for a protein fold family to which JAR1 and its homologs might belong (Kelley et al., 2000). The World Wide Web–based server uses sequence profiles built from query homologs, secondary structure elements, and residue solvent accessibility in a fold recognition algorithm to generate a list of possible matches with the 3D-PSSM fold library. Structural models of possible matches were viewed and analyzed further with the Chime 0.99 browser plug-in (MDL Information Systems, San Leandro, CA) and the molecular graphics visualization software RasTop 1.3.1 (Philippe Valadon, La Jolla, CA).

Enzyme Assays

Gene coding sequences for expression as glutathione *S*-transferase fusions in pGEX-4T-1 cells were generated by reverse transcriptase–mediated PCR from mRNA obtained from 3-week-old seedlings using appropriate gene-specific primers. Expression in *Escherichia coli* BL21 cells, cell sonication, and protein purification on glutathione-agarose were performed in 140 mM NaCl, 2.7 mM KCl, and 10 mM Na_2HPO_4, pH 7.3, according to the manufacturer's protocols (Amersham Pharmacia). Elution of bound fusion proteins for gel analysis was performed by boiling in SDS-PAGE loading buffer, and 12% SDS-PAGE minigels (Bio-Rad) were prepared and run according to the manufacturer's protocols. Activity assays were performed in a total volume of 20 μL in 50 mM Tris-HCl, pH 8.0, 0.7 mM $MgCl_2$, 0.14 mM ATP, 0.07 mM PPi, 1 mM DTT, 100 nCi of ^{32}P-PPi, and 1 mM substrate as indicated. Reactions were started by adding 2 to 6 μg of glutathione-agarose–bound fusion protein and then incubated for 10 min at 25°C. One microliter of each reaction was spotted on cellulose polyethyleneimine sheets, developed in 1.9 M formate and 1 M LiCl, and exposed to x-ray film. ^{32}P-PPi was from DuPont–New England Nuclear.

Accession Numbers

The accession numbers for the JAR1-like family genes mentioned in this article are AF316996 (mouse D11lgp1), AF316997 (human ORF), AE007838 (*C. acetobutylicum* Acyl-CoA thioesterase 1), and AB061221 (*Physcomitrella patens* GH3-like protein 1).

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