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The Jasmonate Signal Pathway

John G. Turner,[1] Christine Ellis, and Alessandra Devoto

School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, United Kingdom

INTRODUCTION

Plant responses to many biotic and abiotic stresses are orchestrated locally and systemically by signaling molecules known as the jasmonates (JAs). JAs also regulate such diverse processes as pollen maturation and wound responses in Arabidopsis. Here we review recent advances in our understanding of how JA biosynthesis is regulated, the signaling functions of different JAs, and how the JA signal may be transduced via an E3 ubiquitin ligase. We also examine how outputs from the JA, salicylic acid (SA), and ethylene signal pathways are integrated in the regulation of stress response and plant development.

We use the term *jasmonate* to include the biologically active intermediates in the pathway for jasmonic acid biosynthesis, as well as the biologically active derivatives of jasmonic acid. These compounds are widely distributed in plants and affect a variety of processes (Creelman and Mullet, 1997), including fruit ripening, production of viable pollen, root growth, tendril coiling, plant response to wounding and abiotic stress, and defenses against insects and pathogens.

The function of JAs in defense was proposed by Farmer and Ryan (Farmer and Ryan, 1992), who provided evidence for a causal link between wounding (as caused by insect herbivores), the formation of JAs, and the induction of genes for proteinase inhibitors that deter insect feeding. In particular, they proposed that wounding caused release of linolenic acid (LA), the presumed precursor of JAs, from membrane lipids. New evidence indicates that JA signaling in plants is generally as proposed by Farmer and Ryan, but more complex than they envisaged. This new evidence indicates that intermediates in JA biosynthesis have distinctive biological activity, that an E3 ubiquitin ligase probably regulates most JA responses in Arabidopsis, and that the JA signaling pathway interacts with other defense signal pathways.

A great deal of what we currently know about JA signaling comes from studies on Arabidopsis and tomato. However, there are several discrepancies between the proposed JA signaling pathways of these species, and it is not yet clear whether these reflect gaps in knowledge or reveal fundamental differences in mechanism. For example, Arabidopsis mutants defective in JA biosynthesis or perception are deficient in defense responses and are male sterile (Feys et al., 1994; McConn and Browse, 1996; Vijayan et al., 1998), whereas tomato mutants apparently defective in JA biosynthesis or perception have deficient defenses but are male fertile (Howe et al., 1996; Li et al., 2001). Similarly, the systemic induction of JA responses in tomato is through the well-characterised systemin signal pathway (Constabel et al., 1995; Ryan, 2000; Ryan et al., 2002), but in Arabidopsis there is no evidence for an equivalent pathway, even though systemic signaling can be demonstrated (Kubigsteltig et al., 1999).

The JA signal pathway involves several signal transduction events: the perception of the primary wound or stress stimulus and transduction of the signal locally and systemically; the perception of this signal and induction of JA biosynthesis; the perception of JA and induction of responses; and finally, integration of JA signaling with outputs from the SA, ethylene, and other signaling pathways.

Perception of the Stimulus and Production of the Signal That Initiates JA Biosynthesis

JA signaling can be induced by a range of abiotic stresses, including osmotic stress (Kramell et al., 1995), wounding, drought, and exposure to "elicitors," which include chitins, oligosaccharides, oligogalaturonides (Doares et al., 1995), and extracts from yeast (Parchmann et al., 1997; Leon et al., 2001). JA biosynthesis in Arabidopsis is also regulated by cues in the developing stamen, where jasmonic acid is required for pollen development. However, we do not yet know how these stresses or developmental cues are perceived. One approach has been to search for the earliest response to stress, which would therefore be a candidate for a component of the stress perception/signal transduction pathway.

A mitogen-activated protein kinase named *WIPK* is transcribed minutes after tobacco is wounded (Seo et al., 1995), and the WIPK protein product is activated (Seo et al., 1999). Jasmonic acid and its methyl ester accumulate in wounded tobacco plants, but do not accumulate in wounded

[1] To whom correspondence should be addressed. E-mail j.g.turner@uea.ac.uk; fax 44-1603-592250.

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transgenic plants, in which expression of *WIPK* is genetically suppressed. This indicates that expression of *WIPK* is required for wound-induced JA biosynthesis. However, the wounded transgenic plants accumulated SA and transcripts of the gene *pathogenesis related protein 1* (*PR1*), indicating that suppression of the JA pathway permits wound induction of the SA pathway (Seo et al., 1995). More significantly, transgenic tobacco plants overexpressing *WIPK* accumulate JA and *proteinase inhibitor 2* (*PIN2*) transcripts (Seo et al., 1999). Apparently therefore, the wound-induced transcription of *WIPK* and activation of the protein product activates JA biosynthesis and suppresses SA-dependent signaling (Figure 1).

Similarly, in Arabidopsis, a mitogen-activated protein kinase named MPK4 is activated 2 to 5 min after wounding (Ichimura et al., 2000). The *mpk4* mutant is dwarfed, has elevated levels of SA, and has constitutive expression of systemic acquired resistance (SAR) and the defense-related gene *PR1* (Petersen et al., 2000). Dwarfing is reduced and *PR1* is not expressed in *mpk4* plants containing the *nahG* transgene encoding a salicylic acid hydroxylase, which reduces salicylic acid level. Significantly, these transgenic plants also fail to express the JA-regulated genes *plant defensin 1.2* (*PDF1.2*) and *thionin 2.1* (*Thi2.1*) after treatment with JA. Assuming that the plants did not contain a low level of SA sufficient to antagonise JA responses (Niki et al., 1998), the result indicates that the MPK4 cascade may simultaneously suppress SA biosynthesis and promote JA perception/response required for induction of *PDF1.2* and *Thi2.1*. Therefore, *MPK4* appears to regulate JA perception/response rather than JA biosynthesis, and would therefore act at a different point in the JA pathway than does WIPK (Figure 1).

Assuming that the antibody that detects MPK4 identifies the same protein as that defined by *mpk4*, these results also indicate that the wound-induced activation of MPK4 is probably too rapid for the activating signal to be newly biosynthesised JA. It is therefore more likely that MPK4 is activated by the primary stress perception/transduction signal, or possibly by the rapid release of JA from endogenous stores (Stelmach et al., 2001). A critical question, therefore, is whether MPK4 is activated by a JA signal alone.

The Arabidopsis mutant *constitutive expression of vegetative storage protein* (*cev1*) was isolated on the basis of con-

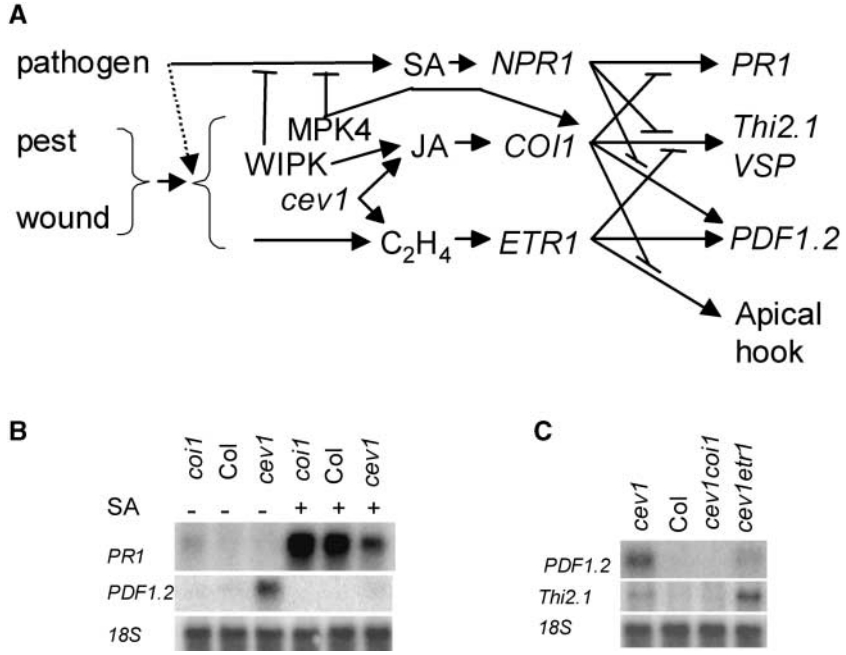

Figure 1. Gene Expression in JA Mutants Reveals Interaction between Defense Signal Transduction Pathways.

Two μg of total RNA from each sample was analyzed on gel blots on nylon filters. Filters were probed with radiolabeled, polymerase chain reaction–generated DNA fragments from *PR1*, *PDF1.2*, *Thi2.1*, and 18S rRNA genes.

(A) Seedlings were grown for 10 days on Murashige and Skoog (MS) agar, then transferred to fresh MS agar (−) or MS agar supplemented with 50 μM SA for 2 further days (+).

(B) Seedlings were grown for 12 days on MS agar.

(C) Model for positive (arrows) and negative (bars) interactions between the JA, ethylene, and SA signal pathways during response to pathogens, and pests or wounding. Gene symbols (in italics) are defined in the text; proteins are upper case, not italic.

stitutive expression of a luciferase reporter for the *vegetative storage protein* (*VSP*) promoter. It is dwarfed, has constitutive production of JA and ethylene, constitutive expression of *PDF1.2*, *Thi2.1*, and the chitinase *CHI*, and has enhanced defenses against fungal pathogens (Ellis and Turner, 2001, Figures 1A and 1B) and an insect pest. The *cev1* mutant phenotype is partially suppressed in the *coronatine insensitive 1* (*coi1*) and in the *ethylene resistant 1* (*etr1*) mutant backgrounds, and the triple mutant, *cev1;coi1;etr1* is wild type except for slightly shorter roots (Ellis et al., 2002). This indicates that *cev1* induces biosynthesis of JA and ethylene, and its mutant phenotype is largely determined by responses to these signaling molecules. *cev1*, therefore, acts at an early step in the stress perception/transduction pathway, before JA and ethylene biosynthesis (Figure 1C). Map-based cloning of *CEV1* identified it as the cellulose synthetase gene *CESA3*. Accordingly, *cev1* had reduced cellulose content, and wild-type plants treated with cellulose synthetase inhibitors have enhanced JA responses and exhibit a near- phenocopy of the *cev1* mutant. Apparently, alterations in the cell wall can initiate JA signaling (Ellis et al., 2002).

When tomato leaves are damaged by herbivores or by simple mechanical wounding, JA signaling and defense gene expression are systemically activated within hours. The systemic signal requires prosystemin, a 200-amino-acid precursor that gives rise to the 18-amino-acid polypeptide systemin by proteolytic processing (Ryan and Pearce, 1998; Ryan et al., 2002). Systemin induces the production of H_2O_2 and the subsequent biosynthesis of jasmonic acid and induction of defense gene expression (Orozco-Cardenas et al., 2001).

Regulation of the Biosynthesis of JAs

JA biosynthesis involves the apparently coincident induction of at least five genes for biosynthetic enzymes, the products of which are targeted to the chloroplast. Gene products for β-oxidation are targeted to the peroxisome, and gene products that modify jasmonic acid are presumably cytoplasmic. The genes for JA biosynthesis are induced at the site of JA formation. Growing evidence indicates that developmentally regulated JA biosynthesis in Arabidopsis is controlled through activation of a JA biosynthetic pathway that differs from, but overlaps with, the biosynthetic pathway that regulates wound-induced JA biosynthesis (Figure 2A).

Release of a-LA

The general pathway for JA biosynthesis presented in Figure 1A indicates that JAs are biosynthesised from the fatty acid LA (18:3). Apparently they may also be biosynthesised from hexadecatrienoic acid (16:3) (Weber et al., 1997). The biosynthesis of 12-oxo-phytodienoic acid (OPDA) from LA occurs in the chloroplast, which contains an abundance of LA esterified in glycerolipids and phospholipids. By analogy with mammalian eicosanoid biosynthesis, a phospholipase A is expected to be responsible for release of LA from membrane lipids. This has recently been confirmed by characterization of the male-sterile Arabidopsis mutant *defective anther dehiscence* (*dad1*) (Ishiguro et al., 2001). *dad1* was isolated from a transposon-tagged population on the basis of its male sterility, which could be rescued by LA or jasmonic acid application. The mutation defined an open reading frame, which encodes a lipase that hydrolyses phospholipids in an sn-1-specific manner, indicating that DAD1 is a phospholipase A1. DAD1 has an N-terminal chloroplast transit peptide, and can accumulate in chloroplasts. The *DAD1* promoter was strongly activated in filaments of stamens prior to the stage at which JA is required for development of the filament, development of pollen grains, and

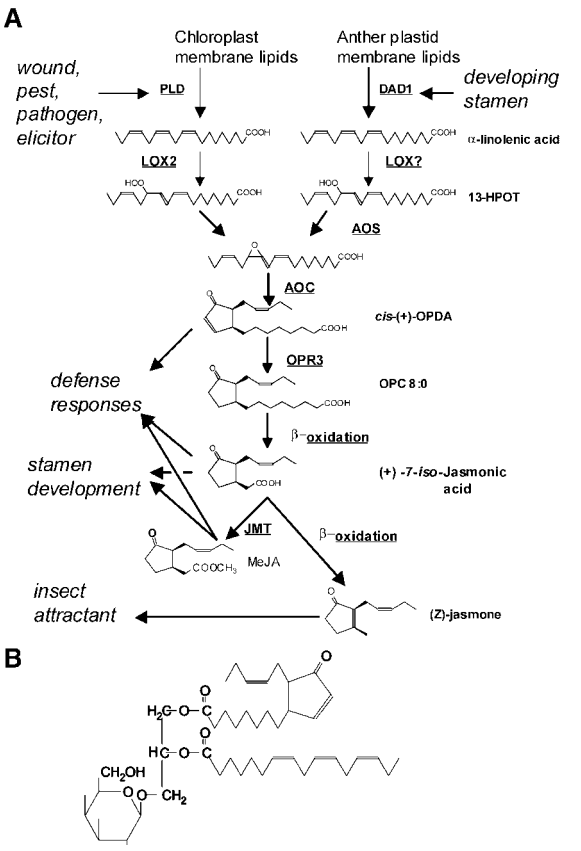

Figure 2. Model for the Biosynthesis of JAs.

(A) Abbreviations for enzyme names are underlined; abbreviations for names of intermediates are in bold; pathway inputs and outputs are in italic.

(B) Structure of sn1-O-(12-oxophytodienoyl)-sn2-O-(hexadecatrienoyl)-monogalactosyl diglyceride, a chloroplast membrane oxylipin containing esterified OPDA.

dehiscence of the anther. These results, therefore, indicate that DAD1 is required for JA biosynthesis in stamen development.

The involvement of DAD1 in wound-induced JA biosynthesis is less clear. Ectopic expression of *DAD1* gave transgenic seedlings with yellow or bleached leaves, indicating that the *DAD1* protein can function in tissues other than anthers. The *DAD1* transcript was maximally induced 1 hr after wounding, but significantly, *dad1* plants were competent for wound-induced JA formation. Therefore, *DAD1* is required for developmentally regulated production of JA for stamen development, and may be involved in, but is not required for, wound-induced JA.

Other chloroplast-localized lipases related to DAD1 have been identified, and one or more of these might be involved in wound-induced JA biosynthesis (Ishiguro et al., 2001). Phospholipase A has also been implicated in wound-, and systemin-induced JA formation in tomato (Narvaez Vasquez et al., 1999). However, other evidence suggests that a phospholipase D (PLD) α is required for wound-induced JA formation in both Arabidopsis and tomato. For example, wounding produces substantial increases in free linoleic and LAs in wild-type plants, whereas transgenic Arabidopsis plants in which *PLD*α is suppressed by antisense show only a slight increase in linoleic acid and no significant increase in LA (Zien et al., 2001). Suppression of Arabidopsis *PLD* by antisense also reduced wound-induced JA and the JA-inducible gene *vegetative storage protein*. These transgenic plants were male fertile, however. Assuming that the JA content of flowers of the transgenic antisense plants was not reduced, the results indicate that *PLD*α is required for wound-induced biosynthesis of JA but not for JA biosynthesis in stamen development. However, McConn and Browse (1996) observed that the threshold level of LA for male sterility was less than 5% of wild-type levels. Therefore, it is possible that the JA content of flowers was reduced in *PLD* antisense plants, but not to a level required for male sterility.

Lipoxygenase

Lipoxygenases (LOXs) catalyze the oxygenation of fatty acids to their hydroperoxy derivatives (Figure 1A). Those involved in JA biosynthesis include a 13-LOX that produces 13-hydroperoxy-octadecatrienoic acid, a substrate for several enzymes, including the next in JA biosynthesis, allene oxide synthase (AOS) (Schaller, 2001). Elicitor-treated potato accumulates transcripts for a 9-LOX that forms 9-hydroperoxy-octadecatrienoic acid, which is apparently involved in defenses (Gobel et al., 2001). Antisense suppression of an Arabidopsis stroma-localized plastid *13-LOX2* also suppressed wound-induced JA formation (Bell et al., 1995) but did not affect male fertility. Apparently, therefore, this *LOX2* is required for wound-induced JA formation, but is not required for JA-dependent pollen and stamen development. Presumably one of the other Arabidopsis *LOX2* genes is required for JA formation in pollen and stamen development. *LOX2* gene transcripts accumulate in response to JA (Heitz et al., 1997; Hause et al., 1999).

AOS

AOS catalyses the dehydration of 13-hydroperoxy-octadecatrienoic acid to an unstable epoxide, which is thought to be converted to OPDA by allene oxide cyclase (AOC). Because of the acute instability of the epoxide, AOS and AOC are probably linked functionally and physically. We await a knockout mutation in AOS that will clarify the function of this gene in defense signaling and in pollen development. However, there is only a single gene for AOS in the Arabidopsis genome (Kubigsteltig et al., 1999), and we therefore assume that it functions both in wound-induced JA formation and in developmentally regulated JA formation required for stamen development.

Transcription of AOS occurs within 2 hr after tissues are wounded and occurs in anthers and pollen grains (Kubigsteltig et al., 1999). The Arabidopsis AOS promoter is activated by a variety of signals including jasmonic acid, wounding, OPDA, and SA, indicating that regulation of the expression of the AOS protein might exert a major control on JA signaling (Laudert and Weiler, 1998). However, overexpression of Arabidopsis AOS in transgenic Arabidopsis and tobacco did not alter the basal level of jasmonic acid, but when the transgenic plants were wounded, they produced a higher level of jasmonic acid than did wounded control plants (Laudert et al., 2000). In Arabidopsis and in tobacco, therefore, it appears that wound-induced JA is regulated by the supply of substrate to AOS rather than by the amount of AOS. In these plants, the release of LA from chloroplast lipids may therefore represent the key regulatory step in wound-induced JA signaling.

By contrast, ectopic overexpression of flax AOS in transgenic potato delivers a chloroplast-localized AOS protein, and increases the endogenous JA, indicating that in this species the substrate for AOS may not limit JA formation. However, the JA-regulated *pin2* genes were not upregulated in these transgenic potato plants (Harms et al., 1995), indicating that signals in addition to JA may be required for *pin2* expression.

AOC

AOC catalyzes the stereospecific cyclization of unstable allene oxide to (9S,13S)-12 oxo-(10,15Z)-phytodienoic acid. DNA gel blot analysis using a cDNA clone as probe revealed a single gene for AOC in tomato. The AOC protein is localized to the chloroplast by an N-terminus chloroplast transit peptide, confirmed by immunohistochemical methods (Ziegler et al., 2000). The AOC mRNA is expressed at low

levels in stems, young leaves, and young flowers, contrasting with a high accumulation in flower buds, flower stalks, and roots. *AOC* transcripts are transiently induced in wounded tomato leaves, where it is expressed primarily in the vascular bundle tissues (Hause et al., 2000). It may be significant that the localization of *AOC* transcripts in wounded plants is at the site of release of systemin in vascular tissues (Jacinto et al., 1999), where it activates JA biosynthesis.

OPDA Reductase

Arabidopsis OPDA reductase (OPR3) catalyses the reduction of OPDA to 3-oxo-2-(2′(Z)-pentenyl)-cyclopentane-1 octanoic acid (OPC-8:0). Although Arabidopsis contains at least two other *OPR* genes, named *OPR1* and *OPR2*, and the transcription of these is wound induced (Biesgen and Weiler, 1999), their protein products do not catalyze the reduction of OPDA (Schaller et al., 2000). The Arabidopsis mutants *dde1* (for *DELAYED DEHISCENCE 1*) and *opr3* (for *oxo-phytodienoic acid reductase 3*) define different mutant alleles of *OPR3.* The plants are deficient in biosynthesis of jasmonic acid, and they accumulate OPDA when wounded (Sanders et al., 2000; Stintzi and Browse, 2000). OPR3 is probably located in the peroxisome (Stintzi and Browse, 2000), indicating that its substrate, OPDA, is transported from the chloroplast to the peroxisome. The *opr3*/*dde1* mutants are also male sterile, and male fertility is restored by application of jasmonic acid, indicating that development of the stamen and pollen uniquely requires jasmonic acid. Significantly, *opr3* has competent JA defense responses against insect pests (Stintzi et al., 2001). Therefore, although topical application of jasmonic acid and methyl jasmonate (MeJA) induces transcription of defense genes in Arabidopsis, OPDA alone is sufficient for these responses. This raises the question of which of these JAs represents the active signal molecule in planta.

Interestingly, *OPR3* transcripts are induced by jasmonic acid (Mussig et al., 2000), indicating an opportunity for feedback regulation of gene expression (Mussig et al., 2000). Gene expression in *opr3* plants treated with OPDA differs from that in *opr3* plants treated with jasmonic acid. For example, jasmonic acid induces expression of transcripts for three genes including *VSP*, which are not significantly induced by OPDA. Other genes are similarly regulated by both compounds, and a subset of genes is upregulated by OPDA but not by jasmonic acid. Regulation of these latter genes is *COI1* independent, and they presumably are therefore not required for defense against insects or pathogens (Stintzi and Browse, 2000). Taken together, these results provide strong support for earlier studies (Blechert et al., 1997) indicating that OPDA is a signaling molecule in its own right, and has regulatory activity different from that of jasmonic acid.

It may therefore be significant that more than 90% of the OPDA in Arabidopsis leaves is present as a novel lipid, sn1-O-(12-oxophytodienoyl)-sn2-O-(hexadecatrienoyl)-monogalactosyl diglyceride (Figure 2B) in chloroplast membranes. The OPDA can be released from chloroplast membranes enzymatically by sn1-specific lipases, and this could account for the very rapid transient increase in free OPDA and jasmonic acid when leaves are wounded (Stelmach et al., 2001). The endogenous store of this lipid therefore has the potential to rapidly supply OPDA and other JAs for JA signaling.

Formation of Jasmonic Acid by β-Oxidation

OPC-8:0 undergoes three rounds of β-oxidation to form jasmonic acid. This probably occurs in the peroxisome, where enzymes for β-oxidation are known to be located. However, there is little direct evidence for the subcellular localization of this part of the pathway for jasmonic acid formation, which has received little attention in recent years.

(Z)-jasmone is a common component of plant volatiles and is probably formed by a further round of β-oxidation of jasmonic acid. Its release from plants can be induced by damage, for example during insect herbivory. Electrophysiological monitoring of the olfactory system of the lettuce aphid revealed responses to (Z)-jasmone, which functions as an aphid repellent and as an attractant for insects that feed on or parasitize aphids. (Z)-jasmone was also active in plants, inducing the production of volatile compounds that affect plant defense by stimulating the activity of parasitic insects (Birkett et al., 2000).

Methylation of Jasmonic Acid

The methylation of jasmonic acid to MeJA is catalysed by an *S*-adenosyl-L-methionine:jasmonic acid carboxyl methyltransferase (JMT) from Arabidopsis. *JMT* transcripts occur in vegetative tissues and in developing flowers, and accumulate locally and systemically when tissues are wounded or treated with MeJA. Transgenic Arabidopsis overexpressing *JMT* accumulates MeJA without altering jasmonic acid content, expresses the JA-responsive genes for *VSP* and *PDF1.2* constitutively, and displays enhanced resistance to infection by *Botrytis cinerea*. Evidently the expression of *JMT* alone is sufficient to induce some JA-dependent responses, and MeJA can function as an endogenous signal molecule in plant defenses. Moreover, *JMT* can perceive and respond to local and systemic signals generated by external stimuli, including MeJA itself. Because MeJA is volatile, its production by JMT could mediate intracellular and intercellular signaling, and could also function as an airborne signal mediating intra- and interplant communications in defense (Seo et al., 2001).

Regulation of JA Biosynthesis

Microarray analysis reveals that five out of 41 genes responding to JA are JA biosynthesis genes, indicating the existence of a positive feedback regulatory system for JA biosynthesis (Sasaki et al., 2001). This confirms the findings of others, that JAs induce transcription of *DAD1*, *LOX2*, *AOS*, *OPR3*, and *JMT* (Heitz et al., 1997; Laudert and Weiler, 1998; Mussig et al., 2000; Ishiguro et al., 2001; Seo et al., 2001) (Figure 1A). Significantly, wounding and other stresses that elicit JA responses also induce these transcripts. Moreover, transcriptional activation of these genes occurs at the site of JA biosynthesis. JAs therefore appear to be synthesized locally in response to stress cues and developmental cues, and the products of this pathway provide a feedback loop for amplification of the signal. It is not known whether physiologically significant quantities of JAs move between cells and tissues in Arabidopsis, or whether local and systemic signaling involves an as-yet undiscovered signaling molecule such as the peptide systemin from tomato (Ryan and Pearce, 1998). It is possible—though in our view unlikely—that JA biosynthesis and tertiary signaling (as defined above) is confined entirely to the cell receiving the primary stimulus.

Perception of JA and Induction of Responses

Perception of JA

The JA signal is probably transduced by the activation of receptors that bind these molecules; however, no receptors have thus far been identified. Arabidopsis defense responses are induced by both OPDA and by jasmonic acid, whereas *VSP* transcription and stamen development are induced by jasmonic acid but not by OPDA (Ishiguro et al., 2001; Stintzi et al., 2001). This suggests that in Arabidopsis, at least two pathways transduce secondary JA signals, one for recognition of either OPDA or jasmonic acid for defense responses, and one for recognition of jasmonic acid, but not OPDA, for stamen development. Membrane-spanning receptor molecules have been defined by mutants that are insensitive to other signal molecules (Li and Chory, 1997). However, exhaustive mutant screens for insensitivity to coronatine (a structural analog of MeJA) and to MeJA (Staswick et al., 1992; Feys et al., 1994), and a screen for mutants that do not express the *pVSP-luc* transgene in the presence of JA (Ellis and Turner, 2001), have identified only alleles of the genes *coi1* and *jasmonate resistant* (*jar1*). This suggests that either there is genetic redundancy in the types of JA receptor, or that *COI1* and *JAR1* function in JA perception, even though COI1 is an F-box protein (Xie et al., 1998) and JAR1 has similarity to the auxin-induced GH3 gene product from soybean (P. Staswick, personal communication), and neither protein shows homology to previously described plant receptor proteins (Gilroy and Trewavas, 2001). Interestingly, the *jar1* mutations define an open reading frame previously reported as *fin219* that was isolated as a suppressor of *constitutive photomorphogenesis 1* (*cop1*) responsible for a defect in far-red light signaling.

Post-Translational Regulation of JA Responses in Arabidoipsis via an E3 Ubiquitin Ligase

The *coi1*-1 mutant was isolated in a screen for Arabidopsis mutants insensitive to growth inhibition by the bacterial toxin coronatine, which is structurally related to jasmonic acid (Feys et al., 1994) and to OPDA (Weiler et al., 1994). The *coi1* mutants are also unresponsive to growth inhibition by MeJA, are male sterile, fail to express JA-regulated genes for *vegetative storage protein* (*VSP*) (Benedetti et al., 1995), *thionin2.1* (*Thi2.1*), and the *plant defensin 1.2*, (*PDF1.2*) and are susceptible to insect herbivory and to pathogens (McConn et al., 1997; Thomma et al., 1998). Further alleles of *coi1* have also been isolated in screens for resistance to growth inhibition by jasmonic acid, failure to activate the *VSPA* promoter (Ellis and Turner, 2002), and for susceptibility to bacterial disease (Kloek et al., 2001). The *COI1* gene encodes a 66-kD protein containing an N-terminal F-box motif and a leucine-rich repeat domain (Xie et al., 1998). F-box proteins occur in the eukaryote kingdom in organisms from yeast to man, and function as receptors that recruit regulatory proteins as substrates for ubiquitin-mediated destruction. F-box proteins associate with cullin and Skp1 proteins to form an E3 ubiquitin ligase known as the SCF complex (Bai et al., 1996).

An example of how F-box proteins may regulate defenses is revealed by the F-box proteins βTrCP1 and βTrCP2, which regulate NF-κβ activity in man. NF-κβ is an inducible transcription factor involved in immune, inflammatory, stress, and developmental processes. Iκβα binds to NF-κβ and retains it in the cytoplasm of non-stimulated cells. Tumour necrosis factor induces the phosphorylation of Iκβα to form pIκβα, which is then removed by the ubiquitin proteasome system. A key component is the SCF ubiquitin ligase complex, which contains Skp1, cullin-1, and the two homologous F-box/WD40-repeat proteins, βTrCP1 and βTrCP2. This SCF complex attaches ubiquitin, a small protein that marks other proteins for degradation by the proteasome system, to pIκβα. Ubiquitinated pIκβα is then destroyed in the proteasome, and NF-κβ is activated (Yaron et al., 1998; Suzuki et al., 1999).

We show here that immunoprecipitates of epitope-tagged COI1 from transgenic Arabidopsis plants co-precipitate with SKP1 proteins (Figure 3A), and cullin (not shown) confirming that COI1 forms an SCF^{COI1} complex in vivo. COI1 is therefore expected to form a functional E3-type ubiquitin ligase in plants, and an important question therefore is what substrate COI1 recruits for ubiquitination? We anticipate that these substrates will be key regulators of JA responses.

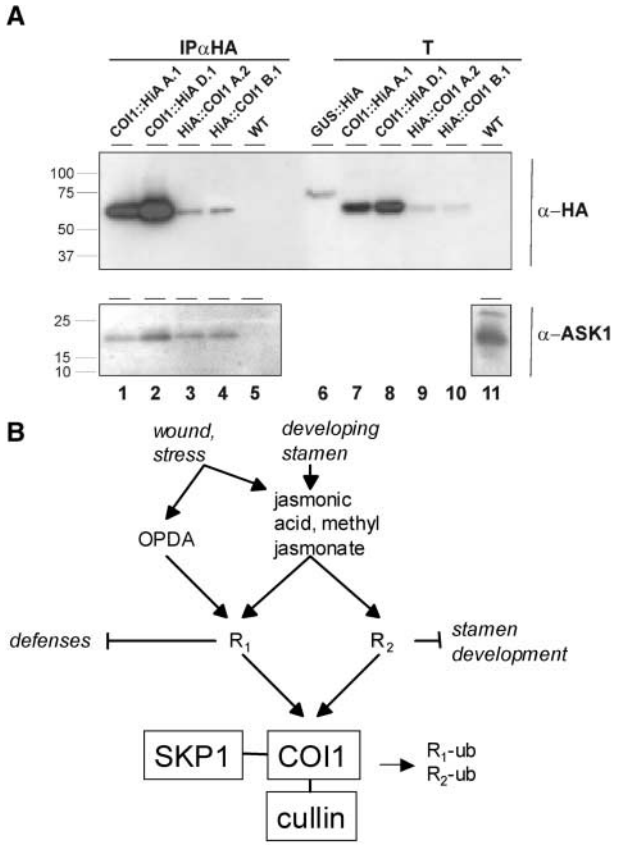

Figure 3. Binding of COI1 with SKP1-like Protein ASK1 in Arabidopsis Indicates that a SCF^{coi1} E3 Ligase Regulates JA Responses.

(A) A protein immunoblot analysis of α-HA immunoprecipitates (IPα-HA; lanes1–5) or total protein extracts (T; lanes 6–11) obtained from MeJA-treated seedlings of independent transgenic *Arabidopsis thaliana* T_2 lines expressing COI1 as haemoagglutinin (HA) carboxy- or amino-terminal fusion proteins (COI1::HA or HA::COI1). Top: detection with monoclonal α-HA antibody (Roche). Bottom: detection with polyclonal antisera raised against Arabidopsis SKP1 (αASK1). GUS::HA: Arabidopsis transgenic lines expressing GUS and HA carboxy-terminal fusion.

(B) A model for how COI1 may regulate JA-dependent defense responses and JA-dependent stamen and pollen development in Arabidopsis, through the E3 ubiquitin ligase–dependent modification of hypothetical repressors R1 and R2 of these two processes.

By analogy to substrates for the F-box βTrCP proteins, it is possible that COI1 mediates the removal of transcription factors tagged by JA-dependent phosphorylation. *JAR1* is required for JA-dependent defenses, but apparently not for stamen and pollen development. Therefore, at least two pathways are regulated by the perception of JAs, one that regulates stamen and pollen development and requires only *COI1*, and another that regulates defenses and requires both *COI1* and *JAR1*. We therefore hypothesize that COI1 regulates two pathways, one for jasmonic acid–dependent stamen and pollen development (which may respond to jasmonic acid and MeJA only), and one for JA-dependent defenses (which may respond, in addition, to OPDA), and we propose a model (shown in Figure 3B) for this phase of JA signaling.

Transcriptional Regulation of JA Responses

JA induces biosynthesis of many classes of secondary metabolites in different species. *ORCA3* is a JA-responsive APETALA2 (AP2)-domain transcription factor from *Catharanthus roseus*. Its overexpression results in enhanced expression of several genes for metabolite biosynthesis and in increased accumulation of terpenoid indole alkaloids (van der Fits and Memelink, 2000). ORCA3 specifically binds to and activates gene expression via a JA- and elicitor-responsive element (JERE) in the promoters of JA-response genes, including the terpenoid indole alkaloid biosynthetic gene *strictosidine synthase* (*Str*). Transcription of *ORCA3* mRNA is rapidly induced by MeJA but is not inhibited by cycloheximide. Cycloheximide also does not inhibit transcription of *Str*, indicating that the JA signal may modify pre-existing ORCA protein, which then activates JA responses by direct interaction with the JERE (van der Fits and Memelink, 2001).

The transcription factor OCRA3 has similarity to the ethylene response binding factors (ERFs), which were originally isolated as GCC box binding proteins from tobacco (Ohmetakagi and Shinshi, 1995). Arabidopsis cDNAs encoding five different ERF proteins (AtERF1 to AtERF5) display GCC box–specific binding activity, and are differentially regulated by ethylene, wounding, cold, high salinity, or drought, via *ETHYLENE-INSENSITIVE2* (*EIN2*)-dependent or -independent pathways. Cycloheximide induces marked accumulation of *AtERF* mRNAs. Thus the *AtERF*s respond to extracellular signals to modulate GCC box–mediated gene expression positively or negatively (Fujimoto et al., 2000). It seems likely that JA responses in Arabidopsis are regulated by *ERF*-like transcription factors, and we anticipate that *ERF*-like genes that are rapidly upregulated by JA will be candidate JA-response factors and candidates for COI1-mediated modification.

Integration of JA Signaling with Other Defense Signal Pathways

The JA pathway regulates response to abiotic stress, defenses against insect herbivores and necrotrophic fungal pathogens and surprisingly, defenses against biotrophic pathogens such as the powdery mildews (Ellis and Turner, 2001); it also regulates developmental processes. Infection of plants with a pathogen that induces necrosis leads to the development of SAR to subsequent pathogen attack. SA is necessary for the full expression of both local resistance and SAR, including PR proteins, and production of secondary

metabolites. Pharmacological experiments suggest that there is negative interaction between responses to pathogens and responses to wounding. For example, silencing the expression of tobacco phenylalanine ammonia lyase (PAL) reduces SAR to *Tobacco mosaic virus* but enhances herbivory-induced systemic resistance to the insect *Heliothis virescens.* Overexpression of PAL enhances SAR but reduces resistance to the insect pest, indicating an inverse relationship between SA-dependent resistance to pathogens and JA-dependent resistance to insect herbivores (Felton et al., 1999). This inverse relationship has been observed in other species also. In Arabidopsis, the *enhanced disease susceptibility 4* (*eds4*) mutation causes enhanced susceptibility to infection by the bacterial pathogen *Pseudomonas syringae* pv *maculicola* and reduces accumulation of SA after infection. The *eds4* mutation also causes heightened responses to inducers of JA-response genes, indicating that SA normally interferes with JA signaling (Gupta et al., 2000).

JA regulates wound responses and defense against insect pests, and is implicated in drought responses. However, microarray analysis of gene expression in wild-type and *coi1* Arabidopsis plants that were wounded, attacked by insects, or exposed to water stress reveals a surprisingly large overlap of *COI1*-dependent genes regulated by wounding and by water stress, and an unexpectedly different profile of genes regulated by wounding and by herbivory (Reymond et al., 2000). The results suggest that some insect herbivores may minimize the activation of a subset of water stress–inducible, defense-related genes. The JA signal pathway also interacts with the ethylene signal pathway in the expression of defense responses and in development. Again, microarray analysis reveals that of 41 JA-response genes, three are involved in signaling pathways for ethylene, auxin, and salicylic acid, confirming the interaction between JA signaling and other signaling pathways (Sasaki et al., 2001).

The *cev1* mutant has been used to investigate interaction between the JA, ethylene, and SA signal pathways. Treatment of *cev1* with SA suppresses expression of *PDF1.2* and enhances expression of *PR1*, though less so than in wild-type plants (Figure 1A). *coi1* mutants, which are deficient in JA perception/response, have slight but significant *PR1* expression, indicating that a *COI1*-dependent signal normally suppresses *PR1* in untreated plants. The double mutant *cev1;coi1* expresses neither *PDF1.2* nor *Thi2.1*, confirming that expression of these genes requires the JA perception/response pathway regulated by *COI1* (Figure 1B). The mutant *ethylene resistant 1* (*etr1*) was used to make the double mutant *cev1;etr1*, in which *PDF1.2* expression was absent, confirming a requirement for an ethylene signal for *PDF1.2* transcription (Ellis and Turner, 2001). Interestingly, *Thi2.1* is constitutively expressed in this double mutant, indicating that ethylene signaling suppresses the transcription of *Thi2.1* (Figure 1B). These results are summarized in a model (Figure 1C) that emphasizes the positive and negative interaction between the JA, SA, and ethylene signal pathways.

In apparent contradiction to the evidence above that JA suppresses SA responses, analysis of some Arabidopsis mutants with constitutive SA responses reveals a pathway in which JA and ethylene signaling are required for SA responses. The Arabidopsis mutant *nonexpression of PR1* (*npr1*) is insensitive to SA, fails to express SA-induced *PR* genes, and has reduced SAR. A screen for suppressor mutations of *npr1* yielded a dominant mutation named *suppressor of SA insensitivity* (*ssi1*), which has constitutive expression of *PR* genes and restored resistance to *P. syringae*. *ssi1* plants accumulate elevated levels of SA but surprisingly, they have constitutive expression of *PDF1.2* also, which is normally induced by JA and ethylene (Shah et al., 1999). The JA content of these plants is not known, however, and *ssi1* may therefore either activate JA biosynthesis or activate the JA perception/response pathway. When SA accumulation in *ssi1 npr1*-5 plants is prevented by expressing the *nahG* gene, all of the *ssi1* phenotypes are also suppressed, including the expression of *PDF1.2*. Treatment of these plants with benzothiadiazole, which mimics the action of SA but is not degraded by salicylic hydroxylase, induces SA responses and remarkably, induces *PDF1.2* expression also (Shah et al., 1999). The results indicate that *SSI1* is a negative regulator of SA biosynthesis and also suppresses SA-dependent induction of *PDF1.2*. This interpretation presents a paradox, however, because induction of *PDF1.2* by SA is not normally observed in wild-type plants.

ssi1 has some similarity to another Arabidopsis mutant with constitutive expression of *PR* genes, named *constitutive PR 5* (*cpr5*). The *cpr5* phenotype is suppressed in the SA-deficient *eds5* mutant, but is only partially affected by the SA-insensitive *npr1* mutant. *eds5* suppresses the SA-accumulating phenotype of the *cpr* mutants, whereas *npr1* enhances it. This indicates that *cpr5* has an SA-mediated, *NPR1*-independent resistance response. However, the *cpr5* phenotype is also suppressed by the *ethylene-insensitive* mutation *ein2* and by the JA-insensitive mutation *jar1.* Evidently, SA-mediated, *NPR1*-independent resistance in *cpr5* requires components of the JA and the ethylene signal pathways (Clarke et al., 2000).

The *cpr5* and *ssi1* mutants not only display enhanced resistance but also develop spontaneous necrotic lesions that also involve the SA-, JA-, and ethylene signaling pathways. Possibly, therefore, these mutant phenotypes are partially phenocopied by the fungal toxin fumonisin B1, which induces apoptosis-like cell death that requires the JA, ethylene, and SA signaling pathways, as evidenced by the absence of fumonisin-induced cell death in *jar1* and *etr1* mutants and in plants containing the *NahG* transgene (Asai et al., 2000).

Interaction between signaling pathways occurs not only in defense but also in development. In dark-grown Arabidopsis seedlings, the hypocotyl is elongated and in addition, forms an apical hook, a tight $\sim$180° curve in the hypocotyls immediately below the cotyledons (Figure 4). In wild type seedlings exposed to ethylene and in the *constitutive ethylene*

response (*ctr1*) mutant the apical hook is exaggerated and the hypocotyl is shorter and thicker, than wild type plants in the absence of ethylene. Development of the apical hook in dark-grown seedlings of mutants in the JA and the ethylene signaling pathways, untreated or exposed to ethylene or JA, is influenced by the balance between ethylene and JA signaling. Thus, JA suppresses the apical hook in wild-type and *ctr1* seedlings but not in *coi1* seedlings, and ethylene enhances the apical hook in wild-type and *coi1* seedlings (Figure 4). The response of the *cev1* mutant to JA and ethylene is similar to that of wild-type plants, and the response of the double mutant *cev1;etr1* is similar to that of *etr1* mutants. These results reveal an inverse relationship between the JA- and the ethylene-signaling pathways on apical hook development (Ellis and Turner, 2002). Therefore, JA and ethylene signaling regulate defense responses and development. Evidently the particular response to these signaling molecules must be determined in part by the physiological poise of the cell upon which they act.

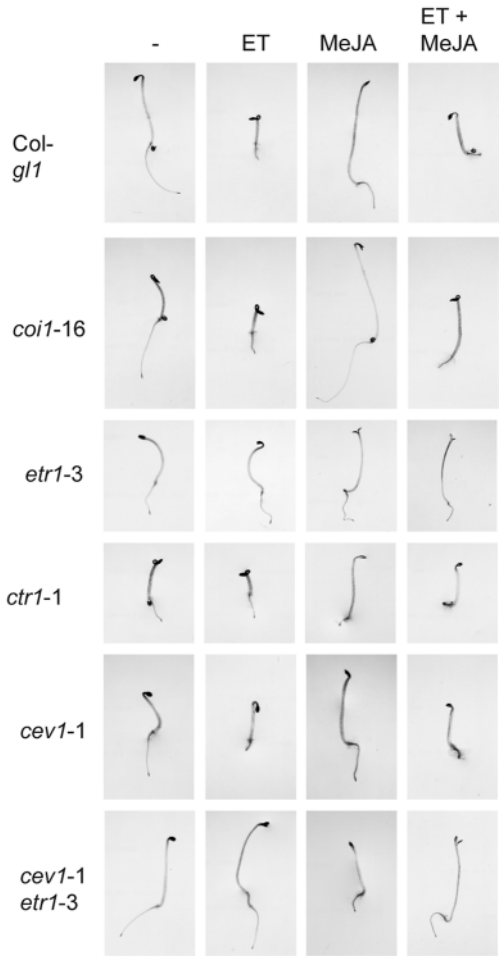

Figure 4. The Effect of MeJA and Ethylene on the Morphology of Arabidopsis Seedlings Indicates Interaction between These Signal Pathways in Development.

Ethylene (ET) induces a pronounced apical hook in wild-type Col-*gl1* seedlings but not in the ethylene-insensitive mutant, *etr1*-3. The mutant *constitutive triple response* (*ctr1*) has an apical hook even in the absence of ethylene. Treatment with MeJA (MeJA), suppresses the apical hook in Col-*gl1* and *ctr1*, but not in the JA-insensitive mutant *coi1*-16. *cev1* has constitutive JA and ET signaling (see Figure 2), and responds appropriately to exogenous MeJA and ET; ET responses in *cev1* are suppressed in the *etr1*-3 background. (−), no treatment.

Novel Mutants with Constitutive JA Responses

Several Arabidopsis mutants with constitutive JA responses, which cannot yet be placed in the JA signal pathway, have been isolated recently. In one ingenious genetic screen, Arabidopsis seed carrying a transgene, consisting of the *bar* gene for resistance to the herbicide BASTA fused to the JA-responsive promoter of the *Thi2.1* gene, were mutagenised, and BASTA-resistant seedlings were isolated. The herbicide-resistant mutants, named *constitutive expression of Thionin2.1* (*cet*), defined five complementation groups with different phenotypes, including enhanced JA and OPDA level, constitutive activation of JA response genes only, constitutive activation of SA and JA responses, and spontaneous necrosis (Hilpert et al., 2001). Xu et al. (2001) isolated the dwarf mutant *constitutive expression 1* (*cex1*), which shows constitutive expression of *PDF1.2*. The mutant dwarf phenotype was not suppressed in the *coi1* mutant background, indicating that it may define a step downstream of *COI1*. In this case, *PDF1.2* expression should also be *COI1* independent, but this critical information is not yet available (Xu et al., 2001). Further analysis of these mutants is likely to reveal novel regulators of JA signaling.

FUTURE PROSPECTS

The JA signal pathway regulates aspects of development and diverse responses to stress. A major challenge is to devise assays that will identify genes that perceive the primary stress signal. In tomato, the primary stress signal is transmitted systemically via peptide signal molecules, but in Arabidopsis, we do not yet how the systemic wound and stress signal is transmitted, and elucidation of this will be a goal for future research. The biochemistry of JA synthesis is relatively well understood, and future work may focus more on the regulation of synthesis. Recent evidence that JA is not transported but is synthesized at the site at which it has effect indicates that characterization of the factors that regulate localized synthesis of JA are fundamental to our understanding of the orchestration of JA responses. We presently do not know how JAs are perceived, and

identification of the JA receptor(s) therefore remains a significant challenge, which has thus far defied biochemical approaches and screens for mutants. Although the JA, SA and ethylene signaling pathways are clearly defined by the signaling molecules they synthesize, they interact cooperatively and antagonistically in a variety of responses. A particular challenge is therefore to discover which points of the JA signaling pathway interact with outputs from the SA and the ethylene signal pathways, and vice versa. Our present understanding of the JA signaling pathway, imperfect as it is, reveals an enormous complexity, and therein the opportunity for multiple control sites and flexibility of function.

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