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BIOCHEMICAL CHARACTERIZATION OF THE COI1-JAZ RECEPTOR FOR JASMONATE

By

Leron J. Katsir

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ABSTRACT

BIOCHEMICAL CHARACTERIZATION OF THE COI1-JAZ RECEPTOR FOR JASMONATE

By

Leron J. Katsir

Jasmonates (JAs) are a class of lipid-derived hormones that regulate diverse aspects of plant development and resistance to environmental stress. The molecular mechanism of JA perception is poorly understood. A central component of JA signaling is the F-box protein COI1 that assembles into the E3 ubiquitin ligase SCF^{COI1}. JAs regulate gene expression by stimulating the ability of SCF^{COI1} to degrade JAsmonate ZIM-domain (JAZ) proteins that repress transcriptional activation of JA responsive genes. I employed an *in vitro* pull-down assay to study the mechanism of COI1-dependent JAZ degradation in response to JA. The results indicate that COI1 physically interacts with JAZ proteins and that this interaction is highly specific for jasmonoyl-isoleucine (JA-Ile) and closely related structures. The bacterial phytotoxin coronatine (COR) stimulated COI1 interaction with tomato JAZ proteins and was at least 100-fold more active than JA-Ile. Testing of a broad range of JA derivatives provided new insight into the structural features of JA-Ile that are required for ligand binding, attenuation of the signal, and the structural basis of COR's enhanced activity. COI1 bears striking sequence and structural similarity to the auxin receptor, TIR1. JA-Ile-dependent binding of COI1 to JAZs is analogous to the role of auxin in promoting the interaction of Aux/IAA proteins with TIR1. Receptor binding

studies showed that COI1 is an essential component of a JA receptor. Analysis of truncated JAZs revealed that the C-terminal domain of JAZ3 is necessary and sufficient for ligand-induced COI1-JAZ interaction and ligand binding. Significantly, binding assays performed with purified proteins showed that neither COI1 nor JAZ alone acts as a JA receptor. Rather, COI1 and JAZ together are required for ligand binding. These findings extend the paradigm of F-box proteins as intracellular receptors of small molecules.

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Biology, synthesis, and perception of jasmonates

1.1. Introduction

Plant speciation is a record of adaptive success in coping with the challenges of a terrestrial existence, like limited access to water, changing temperatures, and solar radiation - not to mention countless hungry heterotrophs eager to consume nutrient-laden photoautotrophic plants. An additional challenge complicating the existence of plants, at least from an anthropomorphic perspective, is that plants are sessile and cannot move to escape from environmental changes, save seed dispersal of the next generation. To cope with a rapidly changing world plants have developed a vast array of sensors to detect changes in the environment and adaptive features to respond to these changes. One such plant sensor takes advantage of the enzymatic generation of oxidized lipids that accumulate when leaf tissue is damaged by various stress conditions. Oxidized lipids as signaling molecules are not unique to plants; in animals the generation of eicosanoids derived by oxygenation of arachidonic acid are regulators of inflammation and immunity. This thesis is primarily concerned with the description of the machinery plants use to recognize a class of oxidized lipid, the jasmonates (JAs).

1.2. JAs regulate a broad array of plant responses

Jasmonic acid is the prototypical member of a family of poly-unsaturated fatty acid (PUFA) derived cyclized oxylipins. JAs constitute a major hormone signaling pathway in higher plants. In unchallenged plants, JA is a minor contributor to overall developmental patterning and progression up until the reproductive

phase. JAs serve a role in developmental regulation, where the hormone is utilized as a cue at the later stages of reproductive patterning (Ito et al. 2008, Mandaokar et al. 2006). Steady state JA synthesis influences a constitutive low-level expression of genes that contribute to a basal level of plant defense. Stress-triggered increases in JA synthesis induce a multi-phased response that reinforces the components of JA signaling and synthesis, initiate the production of a broad range of plant protective compounds, initiate changes in cell cycle progression that effect growth, and promote the generation of signals that redirect plant resources to manage these changes (Devoto et al 2005, Mandaokar et al. 2003, Reymond et al. 2000, Schenk et al. 2000). The sweeping transcriptional reprogramming that occurs in response to increased JA levels can be summarized as a reorganization of plant resources away from growth and towards defense (Howe and Jander 2008).

1.3. Induction of JA synthesis

JA is synthesized rapidly in response to a variety of environmental stimuli, including wounding, cell wall elicitors, osmotic stress, bacterial and fungal pathogens, and UV light exposure (Gundlach et al. 1992, Conconi et al. 1996, Parchman et al. 1997, Kramell et al 2000). How a particular stress signal induces JA synthesis is not entirely clear. The involvement of a lipase that acts to perceive stress and generate free linolenic acid, the primary substrate for JA biosynthesis, is a central tenant of JA signaling. The lipase DONGLE (DGL),

which is a member of the *AtPLA$_1$-I* gene family, may be responsible for initiating JA biosynthesis in vegetative tissue (Hyun et al. 2008). The events that lead to DGL activation have yet to be characterized, however, the generation of phosphatidic acid (PA) has been implicated as a possible signal to activate DGL (Hyun et al. 2008).

More is known about how JA synthesis is initiated in response to developmental cues. JA perception is required for reproductive processes, including pollen development and the maternal control of seed maturation (Xie et al 1999, Li et al 2004, Browse 2005). In Arabidopsis, a phospholipase A_1 (PLA_1) responsible for initiating JA synthesis in floral tissue has been identified as Defective in Anther Dehiscence1 (DAD1). T-DNA insertions in *DAD1* result in anther dehiscence phenotypes observed in JA biosynthetic and perception mutants (Ishiguro et al. 2001). The finding that DAD1 expression is localized to stamens is consistent with the accumulation of JA in floral tissues (Hause et al. 2000). Characterization of DAD1 remains incomplete, and it is not clear if DAD1 acts to release free linolenic acid, OPDA, or both. Induction of JA synthesis in late stamen development is regulated by transcriptional induction of DAD1 by the transcription factor Agamous (Ito et al. 2007). It is possible that a similar sequence of events may lead to lipase activation and JA synthesis in wounded leaves, though this remains to be determined. A deeper understanding of the specificity, sub-cellular localization, and regulation of lipases that generate the JA signal will provide better insight into the mechanics of this process.

1.4. The biosynthetic pathway of jasmonic acid

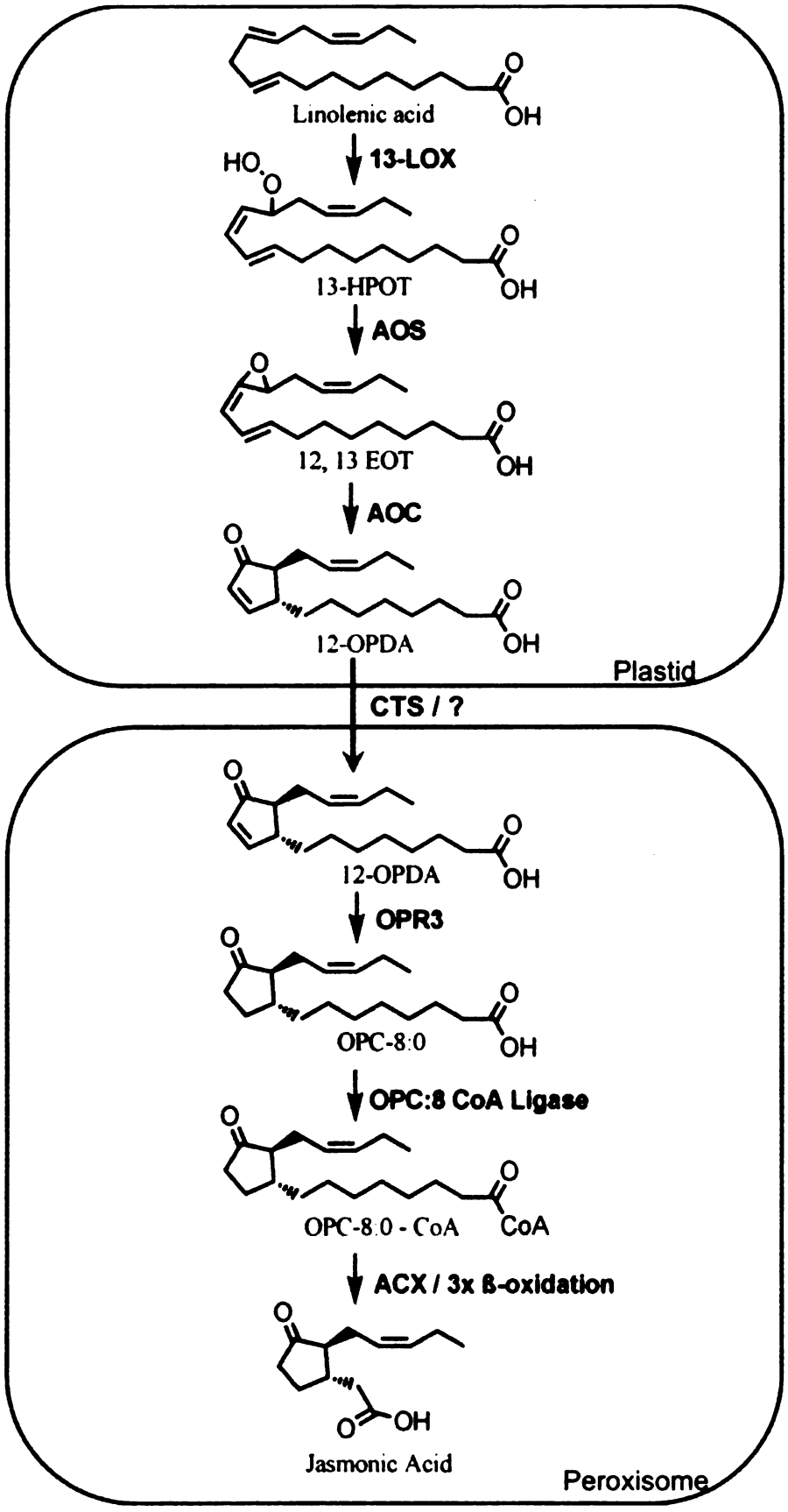
Jasmonic acid is derived from the PUFA linolenic acid. Direct evidence for the role of trienoic fatty acids in JA synthesis came from the work of McConn and Browse (1996), who sought to understand the role of PUFAs in thylakoid membrane function. Instead of finding a photosynthesis-related phenotype in the fatty acid desaturase triple mutant *fad3-2 fad7-2 fad8* that contains <0.1% trienoic fatty acids, they found this mutant to be male sterile. Significantly, fertility could be restored by exogenous JA (McConn et al. 1996). The triple mutant was also more susceptible to insect herbivory, which could be rescued with exogenous JA as well. These key experiments established an important connection between linolenic acid as substrate for JA synthesis (McConn et al. 1997).

The cascade of events that lead to the generation of JA from linolenic acid initiates in the chloroplast where linolenic acid is primarily located and is completed in the peroxisome. Many of the precursors of JA are substrates for the synthesis of other oxylipins (Howe and Schilmiller 2002). An overview of JA biosynthesis is shown in Figure 1.1. In the chloroplast, the initial event the enzyme that acts on linolenic acid (leads to the production of JA) is 13-lipoxygenase (13-LOX), which catalyzes di-oxygenation of linoleic acid to 13-hydroperoxy linolenic acid (13-HPOT). This fatty acid hydroperoxide is then acted on by allene oxide synthase (AOS) to generate an unstable epoxide intermediate 12,13-epoxy octadecatrienoic acid (12,13-EOT). Next, allene oxide cyclase (AOC) guides the unstable epoxide to form the cyclopentenone ring of the more

stable JA intermediate, OPDA. Cyclization by AOC establishes the stereochemistry of OPDA as the (9S,13S) isomer. The detection of OPDA-monogalactosyl diglyceride establishes that a pool of lipid conjugated OPDA exists and accumulates in response to wounding (Stemlach et al. 2001). Determining the role of OPDA-conjugated galactolipids is an active area of research. Regardless, OPDA must be transported to the peroxisome for further processing. The movement of OPDA to the peroxisome may involve the ATP-binding cassette transporter COMATOSE (CTS) and related transporters (Theodoulou et al. 2005).

Figure 1.1 The biosynthetic pathway to jasmonic acid

The enzymes 13-lipoxygenase (13-LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC) catalyze the initial steps of JA biosynthesis in the chloroplast. The ATP-binding cassette transporter COMATOSE (CTS) may have a role in transport of 12-OPDA to the peroxisome. JA synthesis is completed in the peroxisome by the involvement of OPDA reductase 3 (OPR3), OPC:8 CoA Ligase, acyl CoA oxidase (ACX), and additional β-oxidation enzymes (not shown).


Linolenic acid
13-LOX
13-HPOT
AOS
12, 13 EOT
AOC
12-OPDA
Plastid
CTS / ?
12-OPDA
OPR3
OPC-8:0
OPC:8 CoA Ligase
OPC-8:0 - CoA
CoA
ACX / 3x ß-oxidation
Jasmonic Acid
Peroxisome

In the peroxisome, OPDA is reduced to 3-oxo-2- [2'-pentenyl]-cyclopentane-1-octanoic acid (OPC:8) by OPDA reductase (OPR3) (Schaller et al. 2000). The reduction of OPDA is a key step in JA biosynthesis because in the absence of OPR3 jasmonic acid synthesis is aborted. Characterization of *opr3* Arabidopsis mutants led to the idea that some plant-defense responses are stimulated by OPDA rather than JA (Stintzi et al. 2001, Taki et al. 2005). OPC-8:0 then undergoes conversion to its corresponding CoA derivative by the OPC-8:0-CoA ligase1 (OPCL1)(Koo et al. 2006) for entry into β-oxidation. Impairment of β-oxidation, such as in the *acx1/5* double mutant, results in loss of resistance to chewing insects as well as defects in male reproductive development (Schilmiller et al. 2007). JA synthesis is completed after OPC-8:0 undergoes three rounds of β-oxidation (Fig. 1a) (Howe et al. 2005). Jasmonic acid is freed of CoA, presumably by a peroxisomal thioesterase.

Jasmonic acid possesses two chiral centers at the C-3 and C-7 positions, resulting in four possible isomers (Figure 1.2). The stereochemistry of the natural product, *cis* (3R,7S)- jasmonic acid is achieved by AOC. Epimerization of the *cis* compound is the result of a keto-enol tautomerization that results in the more stable *trans* (3R,7R) jasmonic acid (Vick and Zimmerman 1984). Factors effecting epimerization include an acidic environment and temperature, which complicates any analysis of the isomeric composition of jasmonic acid in plants. Neither the rate of epimerization nor the isomeric composition of JA isomers *in planta* is known. The individual activities of these stereoisomers have yet to be carefully evaluated.

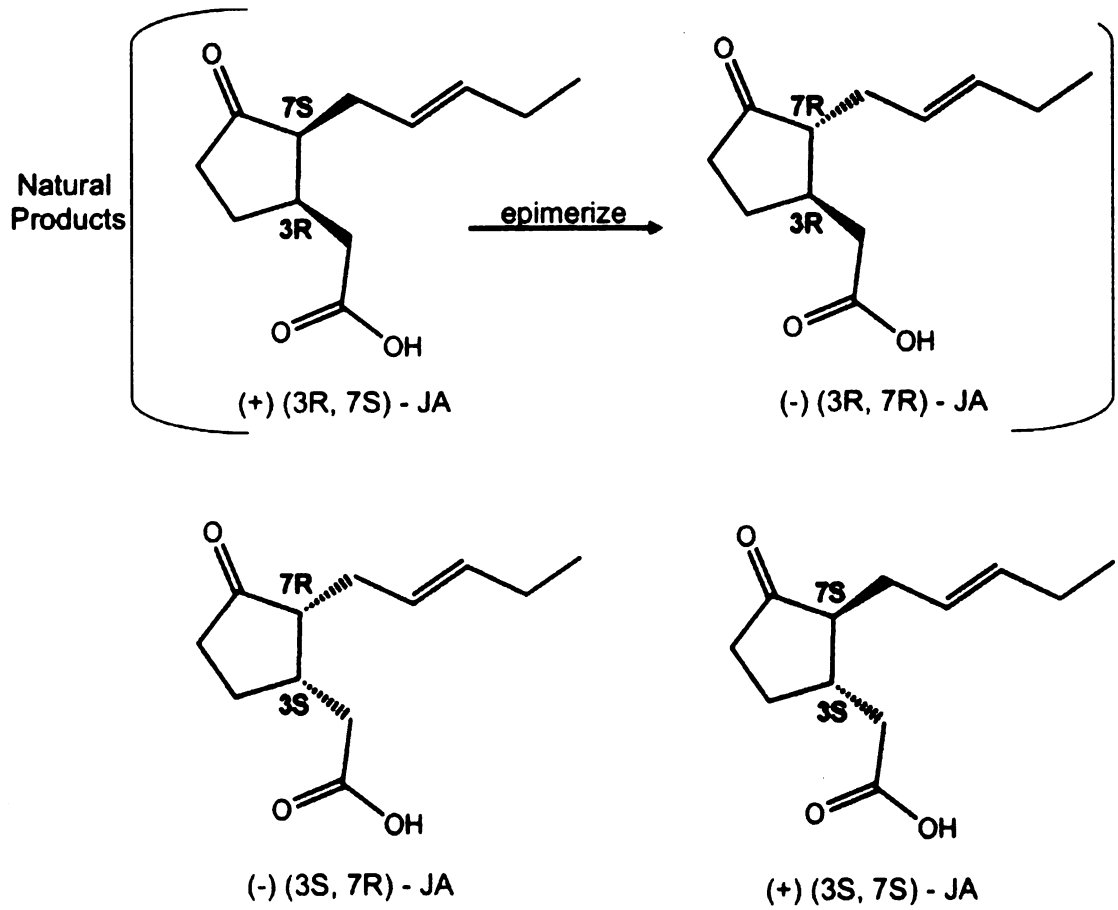


Figure 1.2 Natural and synthetic isomers of jasmonic acid

1.5. Jasmonic acid is metabolized to many derivatives

Newly synthesized jasmonic acid is a substrate for modification of its free carboxylic acid or pentenyl side chain. As such, many derivatives of JA have been identified (see Figure 1.3), yet few have been assigned a definite function. Some JAs have been suggested to affect the mobility, degradation, and/or activity of the JA signal (Miersch et al. 2008).

1.5.1. Hydroxylated JA and related derivatives

Jasmonic acid can be hydroxylated at the 11 and 12-position of the pentenyl side chain (Sembdner et al. 1994, Swiatek et al. 2004, Miersch et al. 2008)(Figure 1.3). Hydroxylated JA can undergo further modification by sulfonation to HSO(4)-JA (Swiatek et al. 2004). An enzyme responsible for sulfonation of 12-OH-JA was identified as 12-OH JA sulfotransferase (Gidda et al. 2003). Wound-induced accumulation of 12-OH JA and HSO(4)-JA compounds lags behind jasmonic acid accumulation, leading to the hypothesis that these derivatives represent JA entry into an inactivation pathway (Miersch et al. 2008). Jasmonoyl-1-β-glucose, jasmonoyl-1-β-gentiobiose and hydroxyjasmonoyl-1-β-glucose are likely produced from 12-OH-JA (Swiatek et al. 2004). The role these compounds serve in plants is not clear.

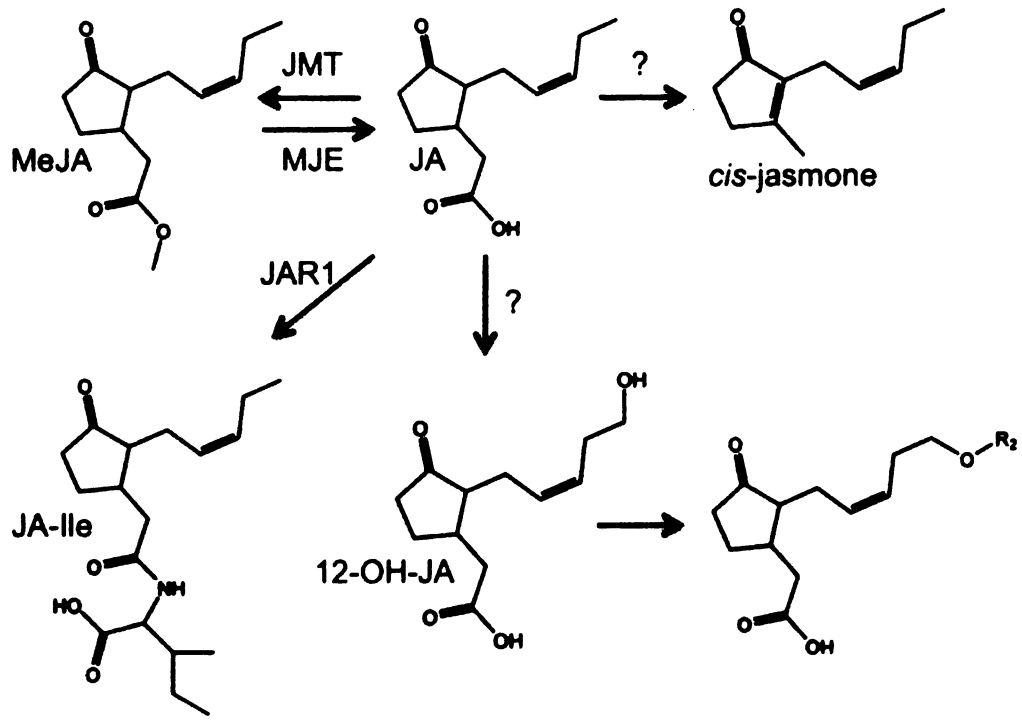


Figure 3.1 Biosynthetic fates of jasmonic acid

Jasmonic acid can be reversibly methylated by the action of jasmonic acid methyl transferase (JMT) and methyl jasmonate esterase (MJE). Conjugation of JA to isoleucine is catalyzed by JAR1. The production of 12-OH-JA from JA can be used as a substrate for addition of a variety of R substituents, including glucose, gentiobiose, and sulfate.

1.5.2. Methyl Jasmonate

The free carboxylic acid moiety of jasmonic acid can be methylated by the jasmonate methyl-transferase (JMT), to yield the more volatile methyl jasmonate (MeJA)(Figure 1.3) (Cheong 2003). Over-expression of JMT up-regulates defense related gene expression and confers increased tolerance to the fungal pathogen *B. cinera* (Seo et al 2001). In tomato, a methyl jasmonate esterase (MJE), that reverses the JMT reaction, was characterized (Stuhlfelder et al. 2005). Because it is more volatile than jasmonic acid, MeJA may be important for movement through plant tissue as a systemic signal (Meyer et al. 2003). MeJA has also been implicated as a plant-to-plant signaling compound that alerts neighboring plants to impending attack (Farmer and Ryan 1990).

1.5.3. *cis*-Jasmone

Feeding of insect herbivore on plants also results in the release of the JA derivative *cis*-jasmone (Birkett et al. 2000). *cis*-jasmone clearly plays a role in plant defense. Though the scope of its activity has yet to be fully evaluated, cis-jasmone has been shown to induce secondary metabolite synthesis and is a potent aphid repellent (Pickett et al. 2007, Birkett et al. 2000). The biosynthetic pathway to *cis*-jasmone is not fully understood. A recent proposal suggests *cis*-jasmone synthesis is initiated by the isomerization of OPDA to *iso*-OPDA. *iso*-OPDA would then proceed through β-oxidation, followed by spontaneous decarboxylation to produce *cis*-jasmone (Dabrowska et al. 2007). An alternative

to the *iso*-OPDA model relies on the spontaneous or enzyme mediated decarboxylation of jasmonic acid to *cis*-jasmone (Figure 1.3). The mechanism of *cis*-jasmone perception is unknown.

1.5.4. Jasmonyl-amino acid conjugates

Jasmonic acid can be conjugated via an amide linkage to amino acids. The jasmonyl-amino acid conjugates (JACs) JA-Ile, JA-Leu, JA-Val, and JA-Phe have been found in a variety of plant species (Kramell et al. 1995, Staswick et al. 2004, Hause et al. 2004). Jasmonyl-isolelucine (JA-Ile) has been proposed to be an active jasmonate because it elicits responses similar to JA (Kramell et al. 1997, Staswick et al. 2004). It is difficult to resolve the activity of exogenous JACs from other JAs because of the occurrence of metabolic inter-conversion *in planta*. The contribution of JACs to JA signaling was reinforced with the discovery and characterization of the Arabidopsis mutant *jar1* that is insensitive to JA but responsive to JA-Ile (Staswick et al. 2002). *JAR1* encodes an enzyme that is structurally similar to members of the firefly luciferase super-family. JAR1 catalyzes the ATP-dependent formation of an iso-peptide bond between the free carboxyl group of JA and the α-amino group of most amino acids and is highly specific for JA (Staswick et al. 2004). Levels of JA-Ile are markedly reduced in the *jar1* mutant, whereas other JACs are less affected, indicating JAR1 is specific for JA-Ile synthesis (Figure 1.3) (Staswick et al. 2004). The phenotypes associated with *jar1* and *JAR* silenced lines of *N. attenuata* demonstrate that JA-Ile synthesis is important for plant protection against necrotrophic soil pathogens (Staswick et al. 1998), lepidopteran insects (Kang et al. 2006), and various

abiotic stresses as well (Rao et al. 2000). However, *jar1* mutants retain some COI1 dependent responses. For example, the mutant is only partially insensitive to jasmonic acid and lacks the reproductive defects characteristic of mutants blocked in JA synthesis and perception (Staswick et al. 2004). The persistence of JACs in *jar1* mutant plants makes it difficult to distinguish JA mediated processes from those facilitated by JA-Ile (Staswick et al. 2004). These recent findings strongly support a role for JA-Ile in activating the JA response but leave open the possibility that jasmonic acid or other JAs are active as well.

1.6. Coronatine is a molecular mimic of JA-Ile

Coronatine (COR) has played an important role in the dissection of the jasmonate pathway and has been long recognized as a molecular mimic of JA (Weiler et al. 1994, Staswick et al. 2008). Most notably, discovery of the *coronatine insensitive mutant1-1* (*coi1)* mutant of Arabidopsis that is insensitive to both COR and JA directly implicates COR in JA signaling (Xie et al. 1998). COR strongly elicits the activation of jasmonate response both locally and systemically (Cui et al. 2005, He et al. 2004, Zhao et al. 2003). Transcriptional profiling of JA- and COR-inducible genes shows that both compounds impact distinct and overlapping pathways, and a large majority of COR action is mediated by COI1 (Zhao et al. 2003, Uppalapati et al. 2005). COR synthesis and secretion is a key element off the virulence strategy of the plant bacterial pathogen *Pseudomonas syringae*. The penultimate step in COR synthesis in *P. syringae* resembles the conjugation of JA and amino acid by JAR1. The

coronafacic acid ligase catalyzes the conjugation of coronafacic acid (a polyketide) and coronamic acid (a cyclopropyl amino acid) moieties of COR (Bender et al. 1999). The resulting COR molecule shares striking structural similarity to JA-Ile (Figure 1.4).

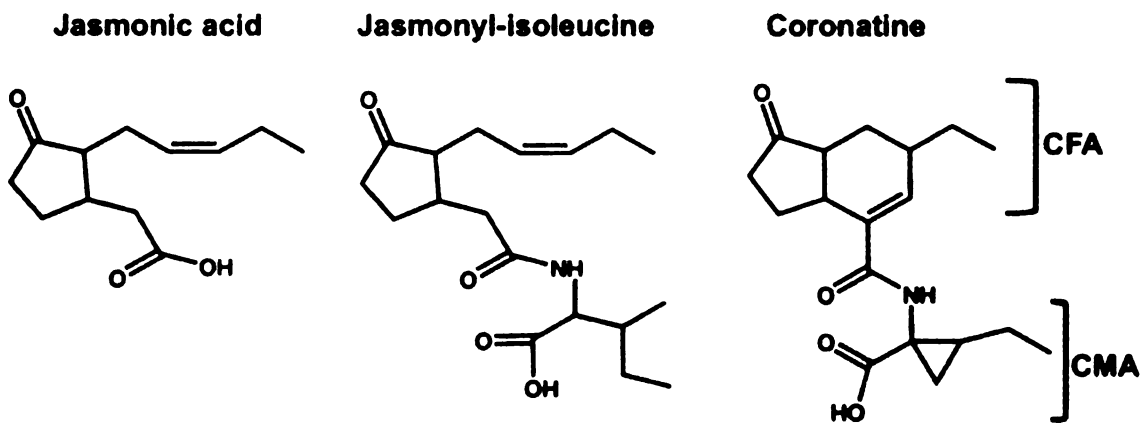


Figure 1.4 The structures of jasmonic acid, jasmonoyl-isoleucine, and coronatine.

Activation of COI1-mediated responses by COR suppresses salicylic acid (SA) accumulation and SA-dependent defense responses targeted against bacterial pathogens (Kloek et al. 2001, Uppalapati et al. 2007). Accordingly, in plants lacking a functional COI1, the JA pathway cannot be activated and SA-mediated

defense responses are not suppressed, making these plants more resistant to *P. syringae* (Zhao et al. 2003). *P. syringae* also uses COR to facilitate host entry by reopening or blocking the closure of stomata that shut in response to pathogen-associated molecular patterns (PAMPs)(Melotto et al. 2007). Thus, in addition to serving as a useful tool to investigate COI1 dependent signaling pathways, COR is an important component of the *P. syringae* arsenal used to subvert host defenses.

1.7. COI1 is a major regulator of JA perception

As the name of the *coi1* mutant implies, these plants are insensitive to the effects of COR, as well as to JA. A screen for JA-insensitive mutants in tomato resulted in the discovery of the jasmonate insensitive1-1 (*jai1-1*) mutant that is defective in the tomato homolog of COI1 (Xie at al. 1998, Li et al. 2004). Characterization of these mutants demonstrated a direct connection between JA perception and COI1 and a key link between JA signaling and proper reproductive development was established. *coi1* Arabidopsis plants are sterile as a result of defects in anther filament elongation, anther dehiscence, and pollen development. The tomato *jai1-1* mutant suffers impairment in embryo development as well as defects in male reproductive development (pollen viability). Mutants lacking a functional COI1 exhibit increased susceptibility to a broad range of biotic and abiotic challenges (Howe and Jander 2008).

Cloning of *COI1* revealed that the gene encodes a ~70 kDa protein consisting of an N-terminal F-box domain and a large C-terminal leucine rich repeat (LRR) (Xie et al. 1998). The F-box domain of COI1 places it within a large super family of over 700 F-box proteins (Gagne et al. 2002). Yeast two-hybrid studies revealed that Arabidopsis COI1 interacts with S-phase kinase-associated protein1 (ASK1), ASK2, a Ring-box1 (AtRbx1), and Cullin (AtCul). The human homologs of these proteins are well-documented components of SCF (Skp-cullin-F-box) type-E3 ubiquitin ligase complexes (Schulman et al. 2000). COI1 likewise assembles with these components to form an E3 ubiquitin ligase designated as SCF^{COI1}. COI1 participation in an SCF complex led to the hypothesis that post-translational control by degradation is central to JA signaling (Devoto et al. 2002, Xu et al. 2002).

1.7.1. The F-box domain of COI1: a link between JA signaling and the 26S proteasome

Ubiquitin ligases covalently attach ubiquitin to a lysine residue on a target protein, committing the ubiquitylated proteins for destruction by the 26S proteasome. Ubiquitin is activated for transfer by an E1-activating enzyme and subsequently transferred to an E2-conjugating enzyme that facilitates the addition of ubiquitin to the substrate of an E3 ubiquitin ligase (Deshaies 1999). The F-box protein confers substrate selectivity to the E3 complex through a diversity of protein-protein interaction domains. In the case of COI1, LRRs are well-documented protein-protein interaction domains (Kobe et al. 2001).

COI1 interacts with the constitutive photomorphogenic-9 (COP9) signalasome (CSN), and defects in JA signaling are exhibited by CSN loss of function mutants (Feng et al. 2003). The CSN is a large multimeric complex that regulates the activity of E3-ubiquitin ligase complexes by removal of the small protein NEDD8 from the cullin subunit of the SCF complex (Chew et al. 2007). Much of our understanding of COP9 action in plants is derived from studying CSN mutants in the context of auxin signaling, where the E3 ubiquitin ligase SCF^{TIR1} is hindered in its ability to facilitate the degradation of its substrate Aux/IAA (Schwechheimer et al. 2001). The similar effects that CSN mutants have on JA and auxin signaling implies that COI1, like TIR1, is dependent on this pathway for proper degradation of key transcriptional regulators. That the CSN impacts multiple hormone pathways reinforces the notion that it is a nexus of hormone regulation via SCF complexes in plants (Serino et al. 2003).

1.8. F-Box proteins serve many roles in hormone signaling

F-box proteins regulate an enormous diversity of plant physiological processes including the regulation of circadian clock, floral development, cell cycle control, leaf senescence, and root and shoot development (Lechner et al. 2006). Several major hormone signaling pathways aside from JA, including gibberellin (GA), ethylene, and auxin are regulated by F-box proteins. Understanding how plants utilize F-box proteins in other hormone signaling pathways may help us understand how COI1 regulates JA responses.

1.8.1. Perception of GA is regulated by $SCF^{GID2/SLY1}$

Gibberellins (GAs) are plant hormones that regulate various developmental processes, including stem elongation, germination, dormancy, and flowering. The GA response is controlled in part by the DELLA proteins, a family of highly conserved proteins defined by the canonical DELLA domain and the VHYNP domain. DELLAs regulate repressors of GA mediated responses. The mechanism by which DELLAs regulate transcription is unknown (Schwechheimer 2008). The F-box proteins GID2 and SLY1, which are involved in GA signaling in rice and Arabidopsis, respectively, confer the specificity for ubiquitin-mediated degradation to the SCF-E3 ubiquitin ligase, $SCF^{GID2/SLY1}$ (Fu et al. 2004, Gomi et al. 2004, Dill et al. 2004, McGinnis et al 2003). The molecular details surrounding GA mediated degradation of DELLA proteins by $SCF^{GID2/SLY1}$ are still being worked out. The GA receptor for GID1 is a enzymatically inactive relative of the so-called hormone-sensitive lipases (HSLs) (Ueguchi-Tananka et al. 2006). Biochemical evidence shows that GA binding GID1 stimulates GID interaction with DELLA proteins (Willige et al. 2007). The model that has emerged involves GID/GA-mediated interaction of $SCF^{GID2/SLY1}$ with, and subsequent degradation of, DELLA. In the absence of DELLA, GA-responsive genes are expressed (Griffiths et al. 2006, Schwechheimer 2007).

1.8.2. The F-box regulators of ethylene perception

Another case of the involvement of an F-box protein in hormone signaling is the gaseous hormone ethylene. Ethylene is involved in mediating responses to biotic and abiotic stress, development, fruit ripening, and senescence (Etheridge et al. 2005). Ethylene is perceived by a family of membrane bound receptors; ETR1, ETR2, EIN4, ERS1 and ERS2 (Hua et al. 1998). These ethylene receptors interact with the serine/threonine protein kinase CTR1 (Kieber et al. 1993). The ethlylene signal is relayed to transcriptional regulators via CTR1 and an unconventional mitogen-activated protein kinase (MAPK) cascade. Most transcriptional regulation in the ethylene response has been attributed to the DNA-binding protein EIN3 (Chao et al 1997). EIN3, and also EIL1 are positive regulators of the ethylene response. EIN3 and EIL1 protein levels are controlled by proteolysis through the 26S ubiquitin proteasome pathway (Guo and Ecker 2003, Potuschak et al. 2003; Gagne et al. 2004). The F-box proteins EBF1 and EBF2 interact with EIN3 and with EIL1 and constitutively target them for degradation (Binder et al. 2007). Ethylene promotes the accumulation of EIN3 and EIL1, thus allowing them to activate transcriptional responses.

1.8.3. The auxin receptor TIR1

The auxin receptor TIR1 is by far the best-characterized F-box protein involved in hormone perception in plants and most relevant to JA signaling. COI1 and TIR1 belong to a small subclade of F-box proteins that is comprised of five additional members, the AFBs (Auxin- signaling F-box protein). All of these

proteins possess a large C-terminal LRR domain (Dharmasiri et al. 2005). The strong sequence (34% identity) and structural similarity between COI1 and TIR1 supports the hypothesis that COI1 is a receptor for JA. TIR1 assembles into an SCF complex, SCF^{TIR1}. Auxin induced-responses are controlled by TIR1-mediated degradation of the Aux/IAA proteins, which act as repressors of auxin signaling (Figure 1.5) (Kepinski et al, 2005, Dharmasiri et al, 2005). Aux/IAA proteins interact with and repress Auxin Response Factors (ARFs) that are positive regulators of auxin gene expression. Auxin mediates TIR1 binding to Aux/IAA by acting as a "molecular glue" to facilitate the interaction of these two proteins (Tan et al. 2008). Bound Aux/IAA is then ubiquitylated and targeted for degradation by the 26S proteasome pathway. Following Aux/IAAs degradation ARFs activate auxin-responsive gene expression. Auxin is the first example of a small molecule that acts non-covalently to enhance the affinity of an F-box protein for its substrates (Kepinski et al, 2005, Dharmasiri et al, 2005). Testing the hypothesis that COI1 acts to regulate JA responses in a manner similar to TIR1 has been hindered by lack of knowledge of COI1 substrates (Figure 1.5).

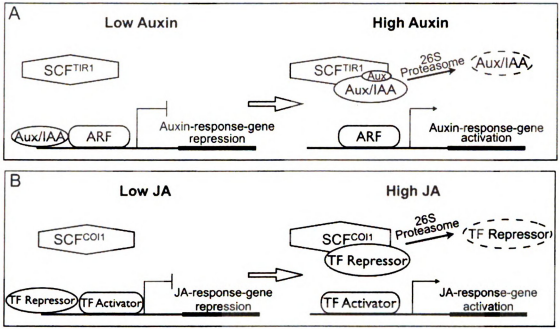


Figure 1.5 Comparison of auxin and JA signaling pathways

(A) The auxin model. In conditions of low auxin concentration, Aux/IAA repressors bind to ARF activators. As auxin concentrations increase, SCF^{TIR1} binding to Aux/IAA is mediated directly by auxin. Aux/IAA is then targeted to the 26S proteasome for destruction. ARF activators are relieved of Aux/IAA repression and activate auxin-responsive genes. (B) The JA model. When JA levels are low, the expression of jasmonate-responsive genes are repressed. An increase in JA concentration leads to SCF^{COI1} ubiquitylation and subsequent ubiquitin mediated degradation of a repressor protein. JA-responsive genes are expressed in the absence of the repressor.

1.9. Transcriptional regulators of JA responses

How JA mediates transcriptional activation through COI1 is unknown. However, several transcriptional regulators have been linked to JA responses. The AP2/ERF-family of transcription factors (TF) is part of the regulatory machinery controlling JA signaling. ORCA2, for example is an AP2/ERF TF that interacts with jasmonate-responsive transcription elements (van der Fits et al. 2001). *ORCA2* transcript rapidly accumulates in response to JA; it has been proposed that the protein plays a primary role in JA responses (van der Fits et al. 2001). The fact that cycloheximide treatment causes the induction of *ORCA2* transcript indicates that its expression is controlled by a labile repressor (van der Fits et al. 2001). Precisely how ORCA2 or other AP2/ERF TFs regulate JA signaling is currently unclear because some members of this family act as positive regulators whereas others act as repressors (McGrath et al. 2005).

The Arabidopsis basic helix-loop-helix (bHLH) TF MYC2 (JIN1) plays a central role in the regulation of JA-responsive gene expression. Arabidopsis plants lacking a functional MYC2 have reduced responsiveness to JA (Lorenzo et al. 2004, Boter et al 2004). MYC2 is a positive regulator of JA signaling and its over-expression results in hypersensitivity to JA (Lorenzo et al. 2004). Tomato homologs of *MYC2, JAMYC2* and *JAMYC10*, have been characterized (Boter et al. 2007). Arabidopsis and tomato *MYC* genes are induced in response to JA treatment. MYC2, JAMYC2 and JAMYC10 proteins also physically interact with - JA responsive promoter elements such as the G-box (Lorenzo et al. 2004, Boter et al 2004). *myc2/jin1* mutant plants do not exhibit the reproductive defects found

in *coi1*, indicating some parts of the COI1-dependent JA pathway are not under the control of this TF. It is noteworthy that MYC2 has also been characterized as a repressor of blue-light mediated photomorphogenic growth and also as a positive regulator of abscisic acid singaling, raising the possibility that this protein mediates crosstalk between several signaling pathways (Yadav et al. 2005, Anderson et al. 2004). Identifying additional transcriptional regulators of JA signaling is essential to understanding how JA influences a broad range of responses.

1.10. Rationale and outlook

Hormone synthesis and perception is fundamental to cellular signaling. The decimation by insect herbivores of plants impaired in JA signaling is evidence of how critical this hormone pathway is to the success of plants (Li et al. 2004, Li et al. 2003). Optimal plant fitness in natural environments depends on the ability of plants to sense and respond to a range of biotic and abiotic challenges. That reproductive development is so tightly linked to plant defense by JA is not surprising considering the pressure to reproduce in challenging environments.

The molecular and biochemical characterization of a receptor is integral to any hormone pathway. The best candidate for such a receptor in JA signaling is the F-box protein COI1. The strong similarities shared between COI1 and the auxin receptor TIR1 make it clear that identifying COI1 substrates would be a breakthrough. The constitutive repression of JA responses in *coi1* mutants supports the existence of such repressor proteins. If the mechanism of JA perception is similar to that of auxin, identification of COI1 substrates would allow experiments to test the hypothesis that COI1 is a JA receptor.

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Chapter 2

Characterization of JA-Ile mediated COI1-JAZ binding[1]

[1] Part of this work has been published in **Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., et al.** (2007). JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. *Nature* , ***448***: 661-5. Yeast-two hybrid assays were carried out by Maeli Melotto.

Abstract

Jasmonate and related signaling compounds regulate a broad range of plant defense responses, as well as growth and development in plants. However the molecular mechanism of jasmonate perception is poorly understood. Recently, members of the jasmonate ZIM-domain (JAZ) protein family were identified as key regulators of jasmonate signaling. Jasmonate treatment causes JAZ degradation that is dependent on the activity of the SCF^{COI1} ubiquitin ligase and the 26S proteasome. Recombinant tomato JAZ protein is used to test the interaction between COI1 and JAZ in an in vitro pull-down assay. The jasmonoyl–isoleucine (JA–Ile) conjugate, but not jasmonic acid, 12-oxo-phytodienoic acid, or methyl-jasmonate, promotes a physical interaction between COI1 and JAZ1 in the absence of other plant proteins. Our results implicate the COI1–JAZ1 protein complex as the site of JA-Ile perception and establish that JA-Ile enables the SCF^{COI1} ubiquitin ligase complex to bind to and subsequently degrade the JAZ1 repressor protein.

Introduction

The synthesis and perception of jasmonic acid, and its bioactive derivatives jasmonates (JAs), is a fundamental component of the biology of higher plants. JAs are regulators of plant growth, reproductive development, and are an integral part of a plants ability to detect and respond to biotic and abiotic challenges (Wasternack 2007, Browse 2005). The JA biosynthetic pathway has been well characterized largely due to the discovery of mutants unable to complete the synthesis of JA. Much of our knowledge concerning JA responses is derived from monitoring transcriptional and physiological changes induced by wounding/herbivory or through exogenous application of JA (Devoto et al. 2005, Mandoakar et al. 2003, Schenk et al. 2000). Such studies have enhanced our broader understanding of how JA responses act to partition plant resources away from growth and towards defense (Howe an Jander 2008). The mechanism by which the JA signal is relayed to produce transcriptional changes is unknown, as a receptor has not been identified. Complicating the search for such a receptor is the diverse array of JAs identified and the *in planta* enzymatic conversions that can obscure the results of hormone application experiments, which confounds efforts to understand, which JAs are active ligands for a receptor.

Characterization of the Arabidopsis *jar1* mutant, which is defective in the perception of JA/Me-JA but responsive to the JA isoleucine conjugate jasmonyl isoleucine (JA-Ile), suggests a central role for JA amino acid conjugates in this

signaling pathway (Staswick et al. 2002, Staswick et al. 2004). In Arabidopsis, the enzyme JAR1 catalyzes the ATP-dependent adenylation of JA and the subsequent formation of an iso-peptide bond between JA's free carboxyl group and the α-amino of most amino acids (Staswick et al. 2004). Though, JAR1 is highly specific for JA and Ile, it is only partially responsible for the production of JA-Ile, as residual JA-Ile remains in the *jar1* mutant. The importance of JA-Ile synthesis to the defense response is reflected in the defects associated with *jar1* plants susceptibility to the pathogens *Pythium irregulare* and *Pseudomonas syringae* (Staswick et al. 1998, Laurie-Berry et al. 2006). JA-conjugate production has also been demonstrated to be a key element in the *Nicotiana attenuata* defense response because silencing of a *N. attenuata* JAR1 homolog, JAR4, impairs herbivore resistance (Kang et al. 2006). Still *jar1* plants are only partially insensitive to JA, and do not share the reproductive defects associated with a block in JA signaling as in coi1 or OPR3. Because of residual JA-Ile in *jar1* plants it is unclear if the remaining JA responses in the mutant are due to the conjugating activity of a redundant enzyme or because other JAs such as jasmonic acid have a distinct activity.

Several lines of evidence indicate that the F-box protein COI1 has a central role in the perception of JA. *coi1* mutants in Arabidopsis and tomato (*jai1*) are unresponsive to JA, are susceptible to a broad range of insect herbivores and fungal pathogens, and have reproductive defects similar to JA biosynthetic mutants (Xie et al 1998, Devoto et al. 2002, Li et al. 2004, Adie et al. 2007). Yeast two-hybrid interaction assays demonstrated that COI1 assembles into an

SCF (Skp, Cullin, F-box) complex, SCF^{COI1}, suggesting that post-translational control by degradation is involved in JA signaling (Devoto et al. 2002, Xu et al. 2002). Furthermore, COI1 shares 34% amino acid identity with the auxin receptor TIR1 (Dharmisiri et al. 2005, Kepinski et al. 2005). Auxin induced responses are controlled by TIR1-mediated degradation of Aux/IAA proteins which act as repressors of auxin signaling (Gray et al. 2002). Auxin mediates TIR1 binding of Aux/IAA by acting as a "molecular glue" to facilitate the interaction between these two proteins (Tan et al. 2008). TIR1-bound Aux/IAA is then presumably ubiquitylated and targeted for degradation by the 26S proteasome pathway. COI1 has been proposed to act similarly to regulate JA signaling (Dharmasiri et al. 2005).

JAZ proteins are transcriptional repressors of JA signaling and were recently identified as substrates for SCF^{COI1} (Chini et al. 2007, Thines et al. 2007). *JAZ* transcripts rapidly accumulate in response to JA treatment. Rapid expression of these genes suggests that JAZ proteins may negatively regulate their own transcription (Thines et al. 2007). The repressive action of JAZ proteins appears to result from interaction with transcriptional activators. This idea is based on the finding that Arabidopsis JAZ3 (also known as JAI3) interacts with and presumably represses the activity of the transcription factor MCY2 (also known as JIN1) (Chini et al. 2007).

JAZ proteins from all plants show overall sequence similarity and possess two signature motifs. JAZs contain the highly conserved TIF[F/Y]XG sequence located within a so-called ZIM domain making them members of the larger family

of TIFY proteins (Vanholme et al. 2007). The TIFY family includes the PEAPOD (PPD) proteins that regulate leaf development (White et al. 2006), as well as ZIM and ZIM-like proteins that other members contain zinc-finger DNA binding domains (Shikata et al. 2003). Although some JAZs are known to be located in the nucleus they do not contain a known DNA binding domain (Chini et al. 2007, Thines et al. 2007). A distinguishing feature of JAZs is the C-terminal sequence $SLX_2FX_2KRX_2RX_5PY$, known as the Jas motif (Chini et al. 2007, Thines et al. 2007). The C-terminal truncated forms of JAZ1 and JAZ3 lack the Jas motif, do not interact with COI1, and strongly repress the JA response in a manner reminiscent of *coi1* (Chini et al. 2007, Thines et al. 2007).

In this study, we identified tomato homologs of JAZ transcriptional repressors. The inducible expression of these genes is similar to that observed in Arabidopsis and other plants. We have developed an in vitro pull-down assay to investigate the mechanism of JA-induced COI1-mediated degradation of JAZ repressor proteins. We present evidence that COI1 directly binds JAZ and that this interaction is dependent on the presence of JA-Ile. Our results support the conclusion that a COI1-JAZ complex is the site of JA perception in plants.

Materials and Methods

Plant Material, Growth Conditions, and Isolation of *jai1-1*

Tomato (*Lycopersicon esculentum*) cv Micro-Tom was used as the "wild type" (WT) for all experiments except in the Northern blot analysis, in which cv Castlemart was used as the wild type. Homozygous *jai1-1* seedlings were selected from F2 populations as described previously (Li et al. 2004). Plants were grown in Jiffy peat pots (Hummert International, Earth City, MO) and maintained in growth chambers under 17 h of light (300 μE m^{-2} s^{-1}) at 28°C.

Blast and Phylogenetic Tree

Arabidopsis and tomato JAZ sequences were aligned with CLUSTALX (Thompson et al. 1997). A phylogenetic tree was constructed with the Neighbor Joining (NJ) method using MEGA 4 software (Molecular Evolutionary Genetics Analysis) available at http://www.megasoftware.net/index.html.

Northern Blot Analysis

Three-week old wild type (WT) and *jai1* tomato plants were subjected to mechanical wounding by crushing the leaf twice across the midrib with a hemostat. For each time point leaf, tissue from a set of three plants was harvested and pooled. cDNA probes were made by using SP6 (5'-ATTTAGGTGACACTATAG-3') and T7 (5'-TAATACGACTCACTATAGGG-3') primers to amplify the cDNA of tomato *JAZ* EST clones cLEC66E8 (SlJAZ1),

cLED-30-L12 (SGN-U319732), cLEC-15-K4 (SlJAZ3), cTOD-21-I17 (SGN-U320368), cLEX-12-K20 (SGN-U327035), and TUS-19-D7(SGN-U315857). EST clone cLED1D24 was used to make a cDNA probe to *eiF4a*, which was used as a loading control. RNA extraction and gel-blot analyses were performed as described previously (Li et al. 2002).

Construction of *35S:COI1-Myc* transgenic tomato plants

The full-length tomato *COI1* cDNA (Li et al. 2004) was amplified by PCR with the primer set of 5'-CGGGATCCCTCTCCTCCATCTTCAA-3' and 5'-CCCTCGAGCTTCAGCGAGAAGGTAAGTTG-3', and the resulting product was digested with BamHI and XhoI. The 6x *Myc* cassette in vector pGEM-72f was obtained from the Arabidopsis Biological Resource Center (ABRC) as stock CD3-128. An XhoI-SacI restriction fragment from this vector was used to generate a 6x-*c*-Myc tag at the C-terminal end of COI1. The *COI1*-containing BamHI-XhoI fragment and *c-Myc*-containing XhoI-SacI fragment were simultaneously ligated to binary vector pBI121 (Clontech) that was pre-digested with BamHI and SacI. Expression of chimeric *COI1-Myc* gene in the resulting plasmid (pBI-COI1-Myc) is under the control of the Cauliflower mosaic virus 35S promoter.

pBI-COI1-Myc was introduced into *Agrobacterium tumefaciens* strain AGLO and subsequently transformed into the *jai1-1* mutant (cv Micro-Tom) as described previously (Li et al. 2004). A primary transformed line (T0-03) that tested positive for the *35S:COI1-Myc* transgene and exhibited seed set was chosen for further analysis. Restoration of seed production in this line indicated

that *35S:COI1-Myc* complements the female sterile defect of *jai1-1* plants (Li et al. 2004). A T1 line that is homozygous for *35S:COI1-Myc* was identified by analysis of progeny in the T2 generation. Seed from T3 plants of this line was bulked for use in pull-down assays.

Cloning and expression of tomato JAZ1-His

A cDNA for tomato JAZ1 (SlJAZ1) was PCR-amplified from an EST clone (cLEC66E8) obtained from the Solanaceae Genomics Network (Cornell University). The primer set used was 5'-GCGCGGCCGCCGGGTCATCGGAAAATATGGATTCC-3' and 5'-CCCTCGAGAGCACCTAATCCCAACCATGC-3' The 0.8 kb PCR product was digested with NotI and XhoI and cloned into the NotI and XhoI restriction sites of the high-copy expression plasmid pLW01 that was modified by addition of the maltose binding protein (MBP). This derivative of pLW01 was provided by Dr. Michael Garavito (Michigan State University). The final construct for SlJAZ1 expression encodes a fusion protein, referred to as JAZ1-His, in which the N- and C-terminal ends of SlJAZ1 are fused to an MBP and 6x-His tag, respectively. SlJAZ1-His protein was expressed in *E. coli* strain BL21 DE3 according to the following procedure. A single *E. coli* colony was inoculated into 5 mL of LB medium containing 100 μg/mL ampicillin, and the culture grown overnight at 37°C. A 0.5 mL aliquot of this culture was used to inoculate 250 mL TB medium containing 100 μg/mL ampicillin. This culture was grown at 37°C to an OD_{600} of 0.6, at which point isopropyl-thio-β-D-galactopyranoside (Sigma-Aldrich) was added to final concentration of 0.5 mM. The culture was incubated at 37°C (with

shaking at 250 rpm) for an additional 3 hr. Cells were harvested by centrifugation at 5,000 x *g* and the cell pellet was frozen at -80°C. Thawed cells were resuspended in lysis buffer (50 mM sodium phosphate, pH 7.2, 300 mM NaCl, 10 mM imidazole, 1 mM phenylmethyl sulfonyl fluoride) and subsequently disrupted by sonication carried out in an ice bath. Lysed cells were centrifuged at 11,000 x *g* for 10 min and the cleared lysate was applied to a Ni affinity column (Ni-NTA resin). The column was washed with three column volumes of lysis buffer, followed by a final wash with lysis buffer containing 25 mM imidazole. JAZ1-His protein was eluted from the column with lysis buffer that contained 250 mM imidazole. Purified JAZ1-His was dialyzed against 1000 volumes of a solution containing 50 mM Tris-Cl (pH 7.6) and 100 mM NaCl. The purity of JAZ1-His was typically >90% as determined by SDS-PAGE and Coomassie Blue staining.

COI1-Myc Pull down Assays

Leaflets from 2- to 3-week-old *35S::COI1-Myc* tomato plants were ground to a fine powder in liquid N_2. Protein was extracted in homogenization buffer containing 50 mM Tris-Cl, pH 7.6, 100 mM NaCl, 25 mM imidazole, 10% (v/v) glycerol, 0.1% (v/v) Tween 20, 20 mM 2-mercaptoethanol, 10 μM MG132, and the EDTA-free complete miniprotease inhibitor cocktail (Roche). Insoluble debris was removed by centrifugation at 14,000 x *g* for 10 min at 4°C. Each pull-down assay contained ~1 mg total tomato protein and 100 μg recombinant SlJAZ1-His in a total volume of 300 μl. Reactions were incubated, with gentle rocking, for 30 min at 4°C, or at the indicated temperature, in the absence or presence of

various JA derivatives. Following the addition of 80 μl of Ni-NTA Resin (Qiagen), the reaction was incubated for an additional 15 min at 4°C. Ni-NTA resin was recovered by centrifugation on a spin column (Biorad) and washed three times with 250 μL of homogenization buffer. The affinity resin was eluted with 30 μL of a solution containing 250 mM imidazole. The eluted protein was separated by SDS-PAGE on a 12% gel, transferred to PVDF membrane, and probed with an anti-*c*-Myc antibody (Roche). The amount of COI1-Myc recovered in the absence of JA-Ile varied between experiments, most likely as a result of differences in endogenous levels of JA-Ile or other active compounds in the tomato extract. Control experiments showed that COI1-Myc was not recovered from pull-down reactions containing JA-Ile but lacking recombinant JAZ1-His or from reactions containing a maltose-binding-protein-His fusion in place of SlJAZ1-His (data not shown). (±)-Jasmonic acid (J2500) and MeJA (392707) were purchased from Sigma-Aldrich. 12-oxophytodienoic acid (OPDA) was chemically synthesized as previously described (Schilmiller et al. 2007). Jasmonoyl-amino acid conjugates were kindly provided by Dr. Robert Kramell (Halle, Germany). Conjugates were synthesized by reaction of (±)-JA with the corresponding L-amino acid, followed by purification of (-)-JA-L-amino acid isomers by chiral HPLC (Kramel et al. 1997)

Yeast Two-Hybrid Assays

SlCOI1 and *JAZ1* were cloned into yeast two-hybrid (Y2H) vectors (Clontech) pGILDA and pB42AD, respectively. The primer pairs used for cloning were: 5'-GGAATTCATGGAGGAACGGAACTCAACGAG-3' and 5'-GCCCTCGAGCTATTCAGCGAGAAGGTAAGT-3' for *SlCOI1*, and 5'-TTACCCGGGCATGGGGTCATCGGAAAATATGGA-3' and 5'-TTACCGCGGCTAGAAATATTGCTCAGTTTTAAC-3' for *SlJAZ1*. The resulting constructs were transformed into yeast (*Saccharomyces cerevisae*) strain EGY48 (p8opLacz) using the Frozen-EZ yeast transformation II kit (Zymo Research). Transformants were selected on SD-glucose medium (BD Biosciences) supplemented with –Ura/-His/-Trp drop- out solution (BD Biosciences). To assess the interaction between COI1 and JAZ1 proteins, transformed yeast strain was plated on SD-galactose/rafinose inducing medium (BD Biosciences) containing –Ura/-His/-Trp drop out supplement, 80 µg/ml X-Gal, and one of the following chemicals: 30 µM jasmonoyl-isoleucine, 30 µM JA (Sigma), 30 µM MeJA (Sigma), 30 µM OPDA (Cayman Chemical Company), or 10% ethanol (the solvent for all chemicals). Plates were incubated for 6 days at 30°C. Yeast two-hybrid (Y2H) control cultures containing the positive control strain pLexA-53 and the negative control strain pLexA-Lam (Clontech) were also plated on inducing medium for comparison of the colony color.

Results

Identification of JAZ Genes in Tomato

Based on the finding that JA-mediated JAZ turnover in Arabidopsis is COI1 dependent we sought to identify *JAZ* genes in tomato to test the hypothesis that JAZ proteins directly interact with COI1. Full-length Arabidopsis *JAZ* cDNAs were used as templates for BLAST searches of the tomato EST library (www.sgn.cornell.edu). We identified and sequenced 28 EST clones with sequence similarity to *AtJAZ* genes. This search yielded several full-length clones representing seven unique tomato *JAZ* genes (Table 2.1).

Table 2.1 Tomato *JAZ* genes

SGN Unigene	Trivial Name	Sequenced EST
U315030	(SlJAZ1)	TUS-3-J20, cLEC-66-E8
U319732	n.a.	cTOS-14-N20, cLED-30-L12
U312806	(SlJAZ3)	cLED-25-A20, cLEC-15-K4
U320368	n.a.	cTOD-23-H21, cTOD-21-I17
U327035	n.a.	TUS-27-L22, cLEX-12-K20
U315857	Pto-responsive gene 1 (Prg1)*	TUS-19-D7
U317846	n.a.	-

* Gen Bank Accession AF146690, n.a. not assigned

The tomato JAZ proteins possess conserved features including, the TIFYxG sequence and the canonical Jas motif $SLX_2FX_2KRX_2RX_5PY$ (**Fig. 2.1**) Deviation from the conserved TIFYxG motif is found in SGN-U319732 (SIFYxG) and in SGN-U320368 (TMFYxG) (**Fig. 2.1**). Phylogenetic comparison of tomato and Arabidopsis JAZs reveals that the tomato proteins cluster among the AtJAZ proteins (**Fig. 2.2**). Tomato JAZs closely related to AtJAZ8, AtJAZ8, AtJAZ10, AtJAZ11, or to AtJAZ12 were not identified. The lack of a broader distribution of JAZ proteins in tomato may be species specific or a reflection of the limitations of gene identification in the absence of a completed tomato genome. Based on our phylogenetic analysis and sequence similarities, we assigned SGN-U315030 as SlJAZ1 and SGN-U312806 as SlJAZ3. SGN-315857 was previously annotated as Pto responsive gene 1 (Prg1) (Table 2.1). The analysis of tomato *JAZ* genes identified through a search of EST libraries must be considered incomplete until the tomato genome is sequenced.

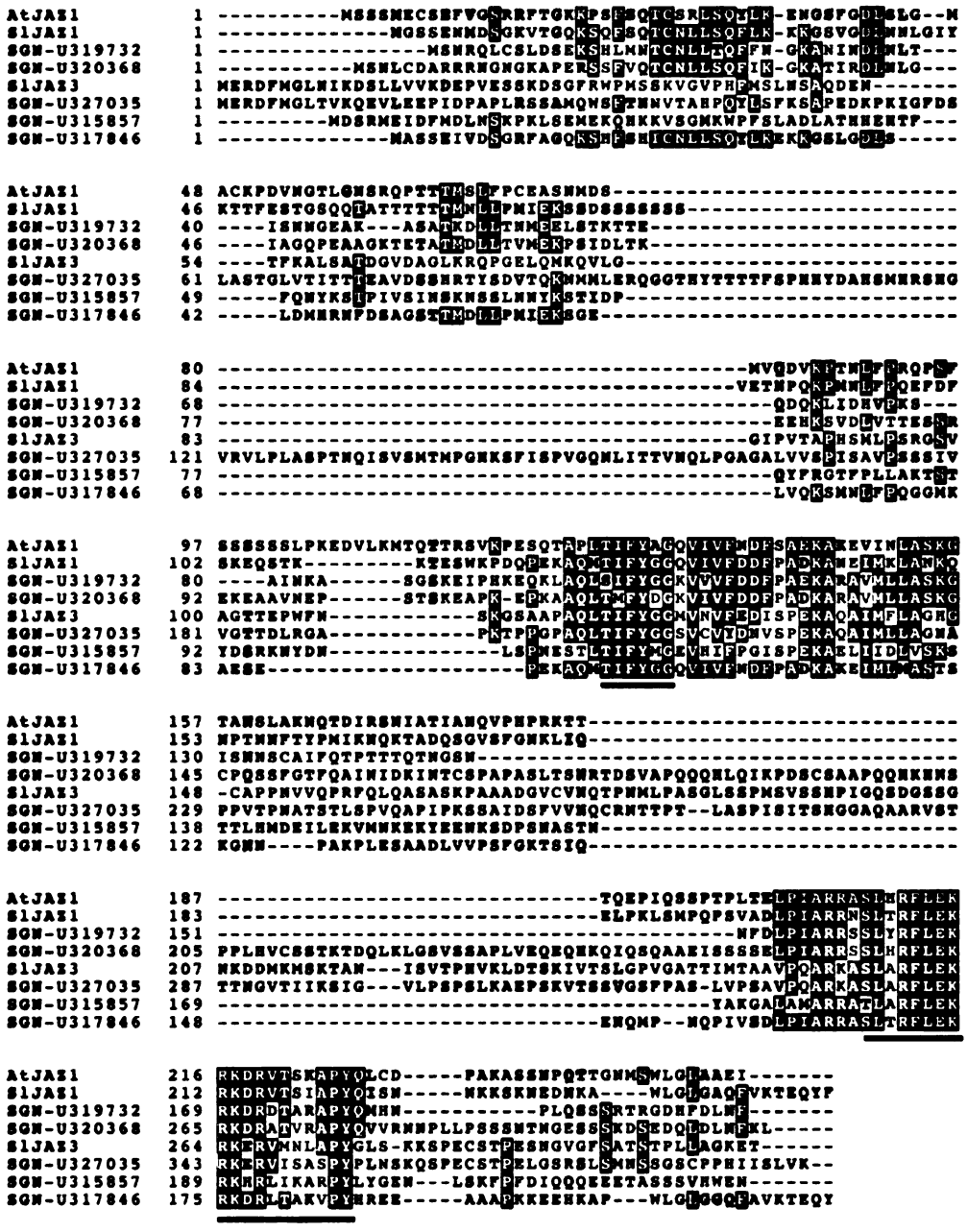

Figure 2.1. Alignment of the 7 tomato JAZ amino acid sequences with AtJAZ1

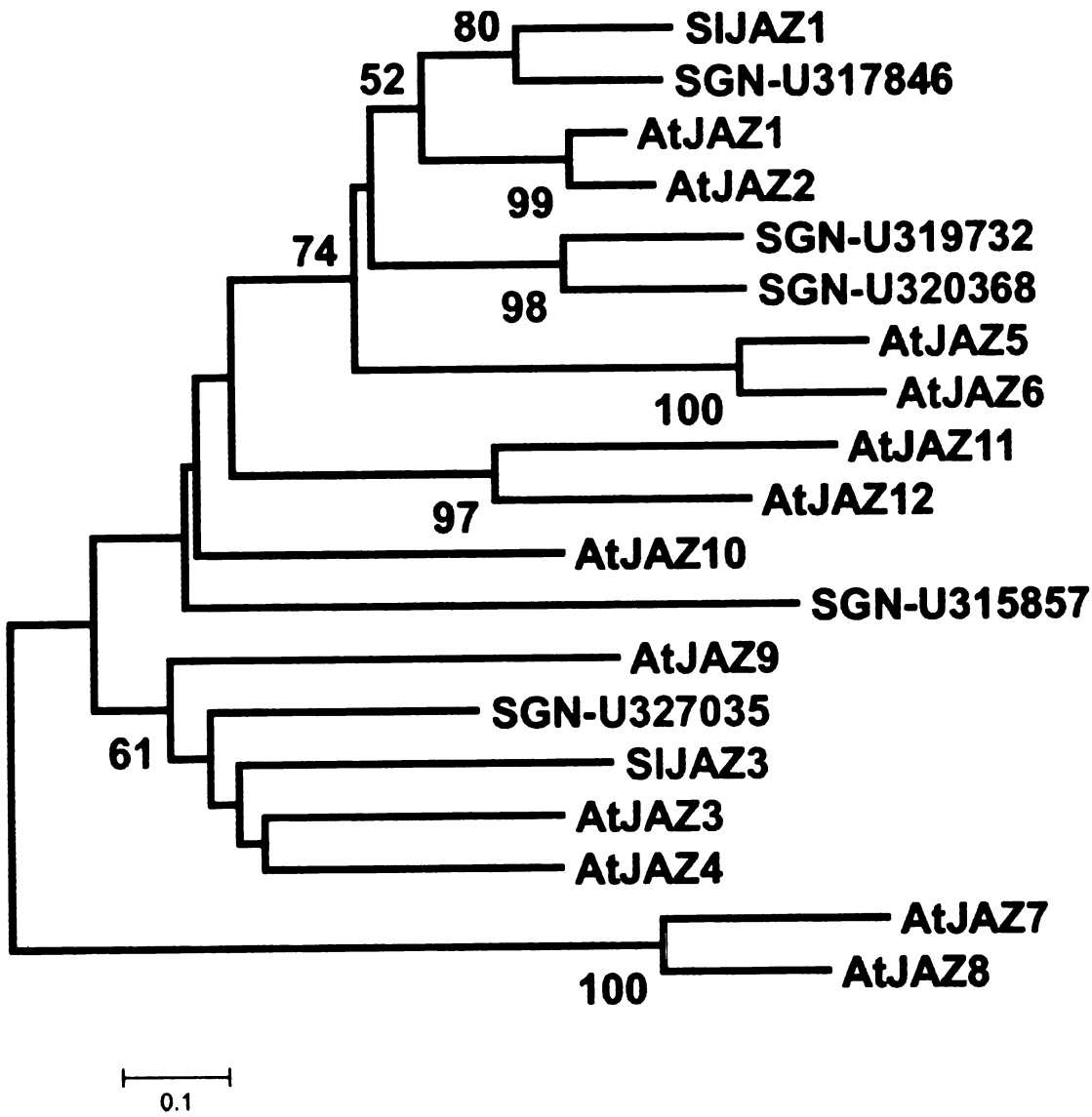
80
SlJAZ1
52
SGN-U317846
AtJAZ1
AtJAZ2
99
SGN-U319732
74
SGN-U320368
98
AtJAZ5
AtJAZ6
100
AtJAZ11
AtJAZ12
97
AtJAZ10
SGN-U315857
AtJAZ9
SGN-U327035
61
SlJAZ3
AtJAZ3
AtJAZ4
AtJAZ7
AtJAZ8
100
0.1

Figure 2.2. Phlylogenetic analysis of Arabidopsis and tomato JAZ proteins.

A phylogenetic tree of twelve Arabidopsis JAZ proteins and seven tomato JAZ proteins was constructed with the Neighbor Joining (NJ) method using MEGA 4 software (Molecular Evolutionary Genetics Analysis) available at http://www.megasoftware.net/index.html. 100,000 neighbor-joining bootstrap replicates were analyzed to determine branching order. Bootstrap values greater then 50 are displayed.

Wound responsiveness of tomato JAZ genes

In Arabidopsis, the majority of the 12 *JAZ* genes are induced rapidly in response to herbivory and mechanical wounding (Chung et al. 2008). Chini et al found that most *JAZ* genes are suppressed in the *jai1-3* mutant and in *myc2,* indicating that a functional JA pathway is required for JA inducible *JAZ* expression (Chini et al. 2007). We used northern blot analysis to detect changes in transcript accumulation of six tomato *JAZ* genes after mechanical wounding (**Fig. 2.3**). We detected *SlJAZ1* and SGN-U327035 transcript accumulation above unwounded levels after 15 minutes and after 30 minutes, these genes, as well as SGN-U315857, were highly expressed. Mechanical wounding also led to an increase in the transcript of SGN-U319732 and SGN-U320368, which were weakly detectable after 60 minutes. *SlJAZ3* was not induced early rather transcript initially decreased at 30 minutes and then began to accumulate above

unwounded levels at 60 and 120 minutes after mechanical wounding. We monitored the effects of wounding on *JAZ* transcript accumulation in *jai1-1* plants to determine whether a functional JA signaling pathway is required for *JAZ* gene induction in tomato (**Fig. 2.3**). For the six tomato *JAZ* transcripts tested, none accumulated to appreciable levels after wounding in *jai1-1*. These results demonstrate that wound inducible expression of the tomato *JAZ* genes is largely dependent on COI1 as previously reported for Arabidopsis *JAZ* genes (Chung et al. 2008).

Figure 2.3. Effect of the *jai1-1* mutation on JAZ gene expression in tomato Three-week-old wild-type and *jai1-1* plants were wounded twice across the midrib with a hemostat. Damaged leaves were collected for RNA extraction at the indicated times (min) after wounding. Ten micrograms of total RNA was loaded in each lane and blots were hybridized to full length probes for each of the six tomato *JAZ* genes, as well as *eIF4a* as a loading control.

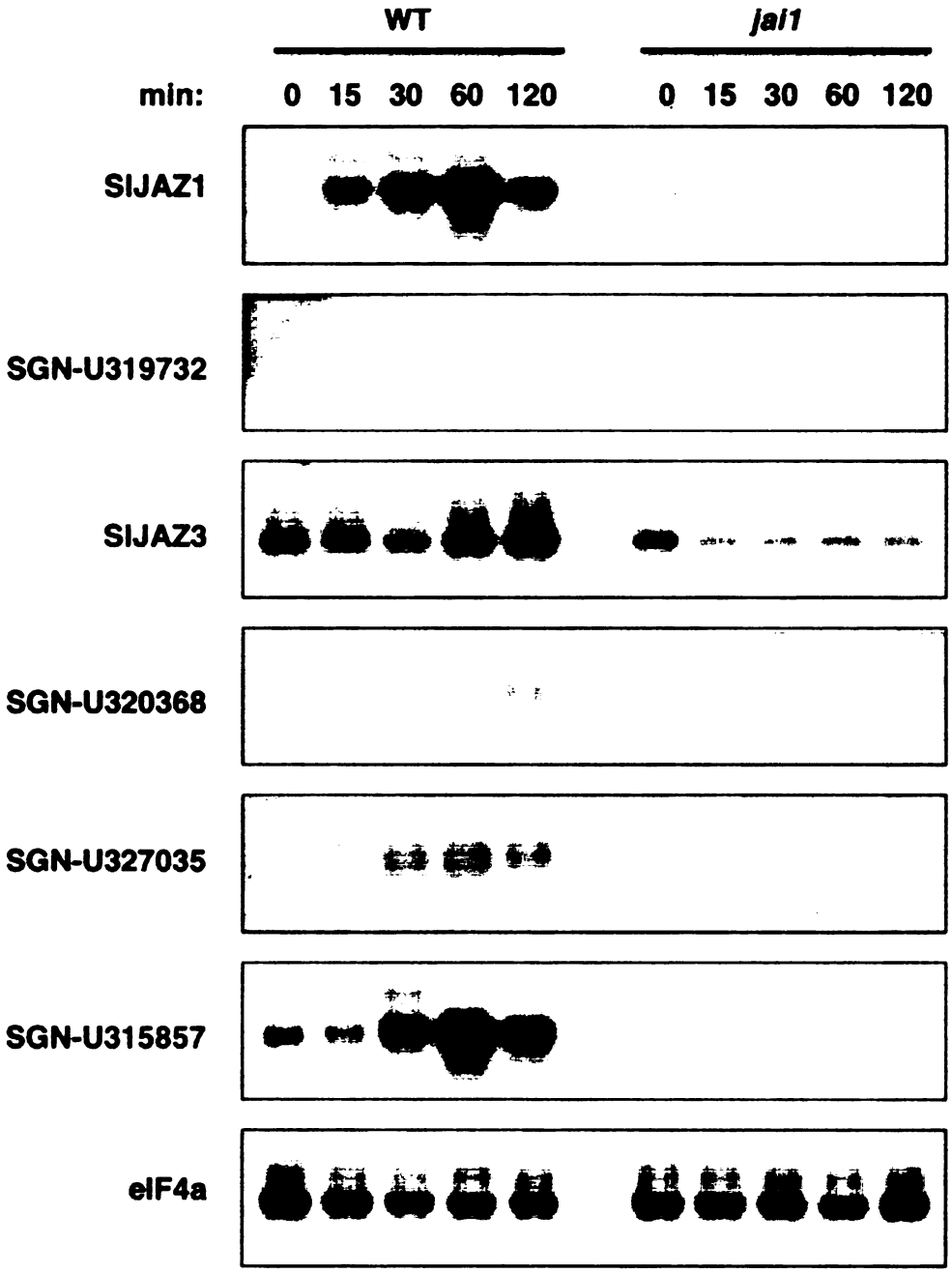

Figure 2.3. Expression of tomato JAZ genes in response to mechanical wounding.

Generation of a transgenic tomato line expressing epitope-tagged COI1

To facilitate the analysis of potential substrates of COI1, the tomato *jai1* mutant was stably transformed with a *35S::COI1-Myc* transgene that encoded a c-Myc tagged COI1 fusion protein, designated. Phenotypic analysis of these lines showed that the defense marker protein proteinase inhibitor II (PI-II) was expressed in response to mechanical wounding (Figure 4A), and that fertility had been restored (data not shown). Complementation of *jai1* by the transgene indicates that COI1 is functional as a fusion with c-Myc. The expression of COI1-Myc in these lines was confirmed by western blot analysis of protein extract with the c-Myc antibody (Figure 2.4B). COI1-Myc expression was not detected by Coomassie staining of total plant protein or in samples enriched for COI1-Myc by anti-Myc affinity resin (data not shown). These findings suggest that COI1-Myc is expressed at very low levels in these plants.

Expression and purification of tomato JAZ1

We next sought to prepare purified JAZ protein for use in pull down assays to test the hypothesis that JAZ interacts physically with COI1. Initial attempts to express tomato JAZ proteins as a His-tagged or GST-tagged fusion in E. coli failed to produce sufficient amounts of protein for use in biochemical experiments. Several other expression vectors were tested, including pB42AD (Clontech) for expression as a glutathione s-transferase fusion, as well as pQE30a (Qiagen) and pet24a (Novagen) for expression of 6X-His tagged

fusions. Finally, expression of tomato JAZ1 as a fusion with maltose binding protein (MBP) at the N-terminus and a 6X-His tag at the C-terminus yielded sufficient amounts of recombinant proteins. The MBP-JAZ1-His fusion, designated simply as JAZ1-His, was purified to ~90% homogeneity by Ni-affinity chromatography (Figure 2.4C).

Figure 2.4. Complementation of the tomato *jai1-1* mutant with *35S:COI1-Myc* and expression and purification of SlJAZ1-His

A, Three-week-old plants of the indicated genotype were mechanically wounded on the lower leaves, and the level of proteinase inhibitor II (PI-II) in upper unwounded leaves was measured 24 hr later (W; grey). PI-II levels were also measured in a second set of plants treated with vaporous MeJA (MJ; white). As a control, PI-II levels were determined in a set of untreated plants (C; black). Data represent the mean and standard deviation of at least 3 plants per treatment. B, Crude extracts (50 μg protein) prepared from leaf tissue of 3-week-old wild-type (WT) and stably transformed *35S:COI1-Myc* (*35S*) plants were separated by SDS-PAGE, blotted to PVDF membrane, and probed with anti-Myc antibody. The apparent *Mr* of the major cross-reacting protein in *35S:COI1-Myc* leaves was in good agreement with the calculated *Mr* of COI1-Myc (approximately 78,000). C, A Coomassie Blue stained gel shows the purity of 10 μg of SlJAZ-His, as determined by SDS-PAGE gel (12% gel) .

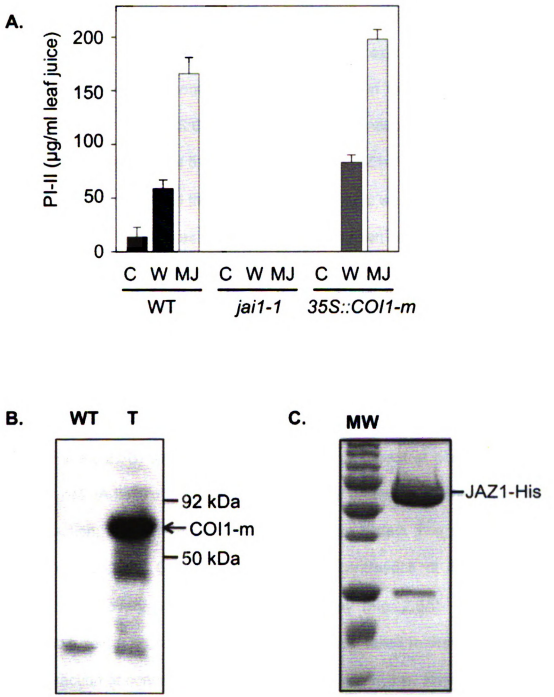

Figure 2.4. Complementation of the tomato *jai1-1* mutant with *35S:COI1-Myc* and expression and purification of SlJAZ1-His

COI1-JAZ1 interaction assay

To determine whether COI1 and JAZ proteins interact in vitro, we took advantage of the *35S::COI1-Myc* transgenic line of tomato that expresses a c-Myc-tagged SlCOI1 (COI1-Myc). We conducted in vitro pull-down assays in which protein extracts prepared from *35S:COI1-Myc* leaves were incubated with recombinant JAZ1-His in the presence of 5 μM MeJA or JA-Ile. JAZ1-His was recovered by Ni affinity chromatography and the presence of COI1-Myc was assessed by western blot analysis. COI1-Myc was recovered from pull-down reactions containing JA-Ile but not from reactions supplemented with MeJA or mock control (Figure 2.5A). The identity of this interacting protein as COI1-Myc was confirmed by its co-migration with COI1-Myc in leaf crude extracts, as well as the absence of this band in control reactions containing protein extracts from wild-type leaves (not shown). JA-Ile promoted the COI1-JAZ1 interaction in a dose-dependent manner (Figure 2.5B) with the stimulatory effect apparent at concentrations as low as 50 nM JA-Ile. To address the specificity of JA-Ile in promoting the COI1-JAZ interaction, a variety of small molecules were tested. The plant hormones auxin, gibberelin, and SA, when added at concentrations of 50 μM, did not promote the recovery of COI1-Myc in this assay (Fig. 2.6A). MeJA, 12-oxo-phytodienoic acid (OPDA), JA-Phe, and JA-Trp also failed to promote the COI1-JAZ1 interaction at concentrations as high as 25 μM. JA-Leu stimulated recovery of COI1-Myc, although its activity was approximately 50 fold less than that of JA-Ile (Fig. 2.6B).

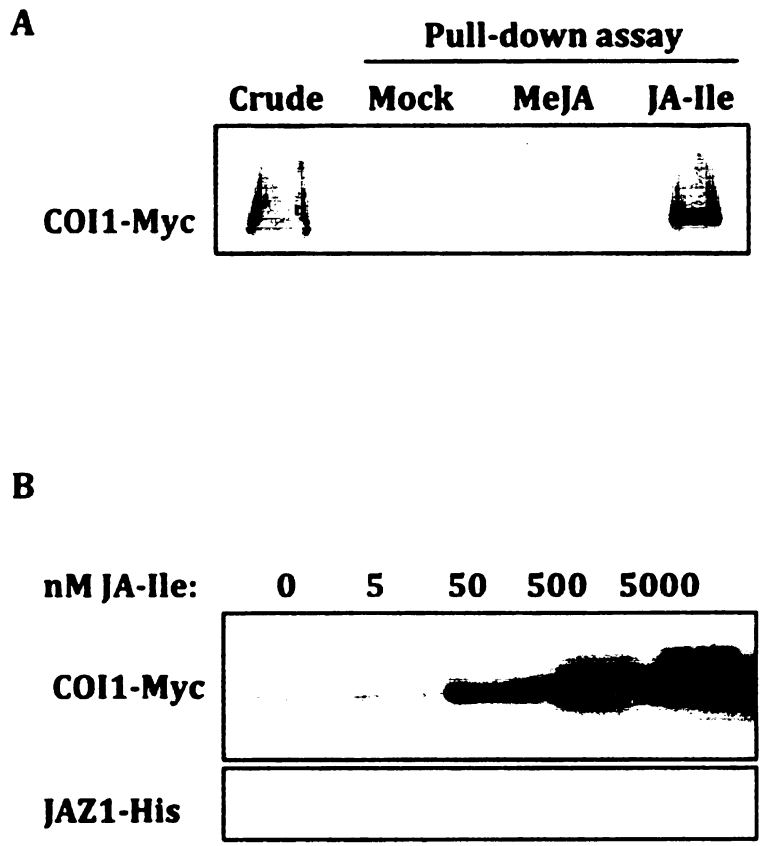

Figure 2.5. In vitro interaction between tomato COI1 and JAZ1 is promoted by JA-Ile.

Pull-down assays used recombinant SlJAZ1-His and extracts from 35S::COI1-Myc plants. A, Reactions were supplemented with 5 μM MeJA, JA-Ile, or a control (mock) and incubated for 30 min at 4°C. Protein bound to JAZ1-His was analyzed by immunoblotting for the presence of COI1-Myc. Leaf extract (crude) shows position of COI1-Myc. B, Assays supplemented with various concentrations of JA-Ile were processed as described above. In B Coomassie stain shows JAZ1-His.

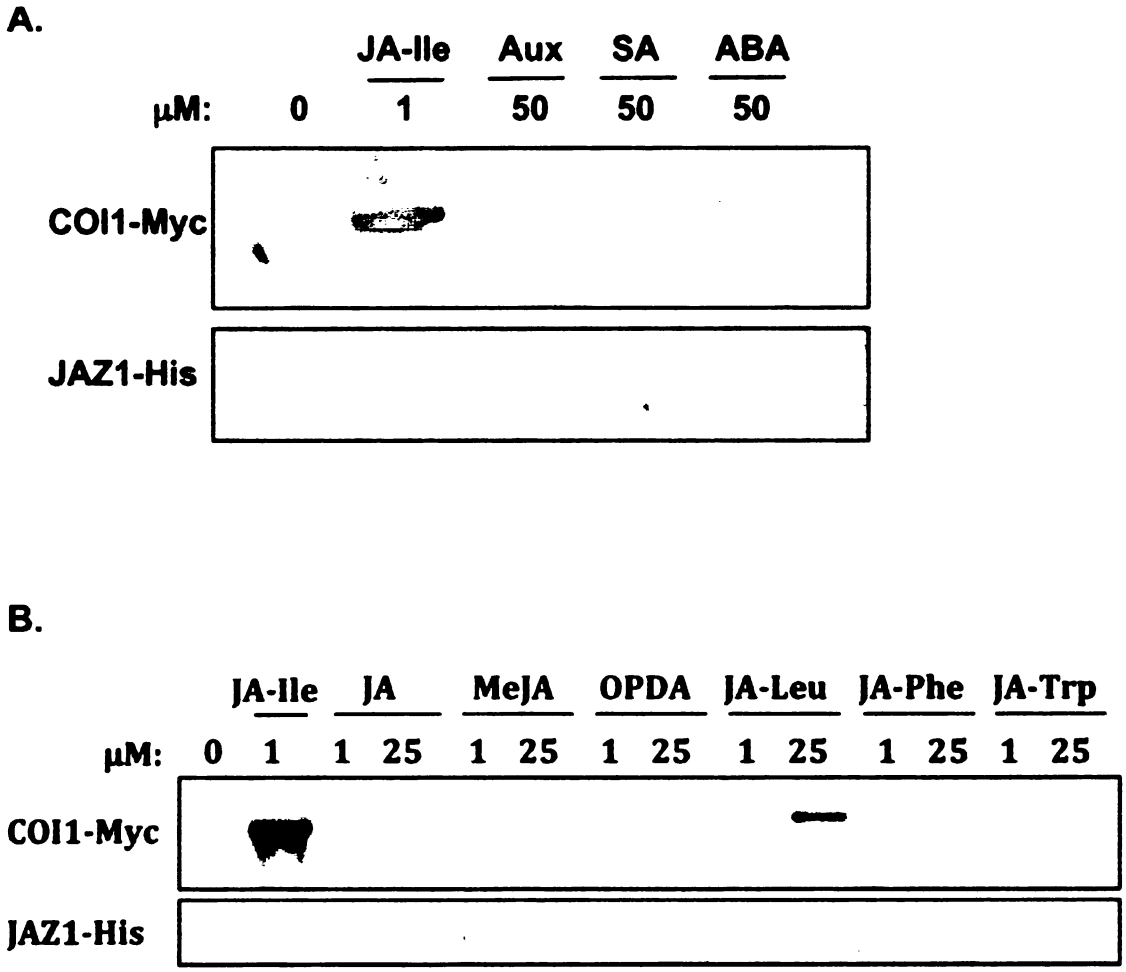

Figure 2.6. Specificity of jasmonate action in a cell-free system.

A, Pull-down reactions containing purified JAZ1-His and protein extract from *35S-COI1-Myc* plants were supplemented with JA-Ile, indole-3-acetic acid (IAA), salicylic acid (SA), abscisic acid (ABA) or an equivalent amount of buffer (Mock) at the indicated concentrations. B, Various jasmonate derivatives were added to pull-down reactions at the indicated concentrations. Reactions were processed as described in Figure 5. The Coomassie Blue-stained blot shows the recovery of JAZ1-His by the Ni-affinity resin.

Characterization of JA-Ile dependent COI1-JAZ interaction

Because these experiments utilized supernatants of tomato leaf extracts, after low-speed centrifugation, as a source of epitope-tagged COI1 (i.e., COI1-Myc), a potential role for cell membranes or membrane-bound proteins in JA-Ile signaling could not be excluded. To test the influence of membrane on COI1-JAZ binding, COI1-Myc-containing tomato leaf extracts were cleared of membrane by ultra-centrifugation. The membrane free extract was tested with the JAZ-His pull down assay to detect the recovery of COI1-Myc. A requirement for cell membrane or an association with it would influence the recovery of COI1-Myc in pull down assays conducted with the membrane-cleared extract. No difference in COI1-Myc recovery was detected in assays supplemented with protein homogenate that had undergone ultra-centrifugation compared to those that had not (Fig. 2.7). This finding indicates that JA-Ile action in the cell-free system is mediated by soluble components.

The involvement of phosphorylation as part of the mechanism of JA signaling has been suggested from studies that link the abrogation of JA-mediated responses to treatment with protein phosphatase 2A (PP2A) inhibitors (Rojo et al. 1998). The stimulatory effect of JA-Ile on the COI1-JAZ interaction may occur indirectly, for example phosphoryl-transfer to COI1 or JAZ, rather than by JA-Ile facilitating the interaction itself. To investigate the possibility that COI1-JAZ binding could be blocked or enhanced by a phosphatase or kinase, we conducted *in vitro* pull down reactions in the presence of the broad range inhibitors of these enzymes.

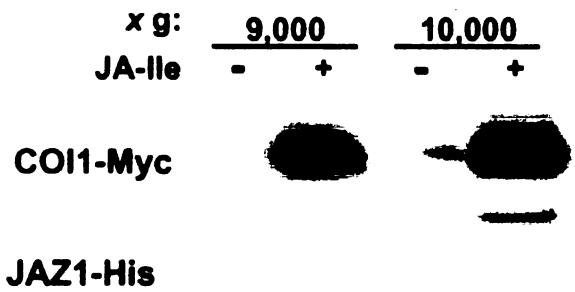

Figure 2.7. JA-Ile-mediated COI1-JAZ interaction *in vitro* is mediated by soluble factors.

Protein extract from *35S-COI1-Myc* plants was centrifuged at 9,000 x *g* for 20 min (9,000) or 100,000 x *g* for 1 hr (100,000). The resulting supernatant was combined with purified JAZ1-His in the presence (+) or absence (-) of 1 μM JA-Ile and incubated for 15 min at 4°C. Following recovery of JAZ1-His on Ni-affinity resin, the presence of COI1-Myc was assayed by immunoblot analysis with anti-Myc antibody. The Coomassie Blue-stained blot shows the recovery of JAZ1-His by the Ni-affinity resin.

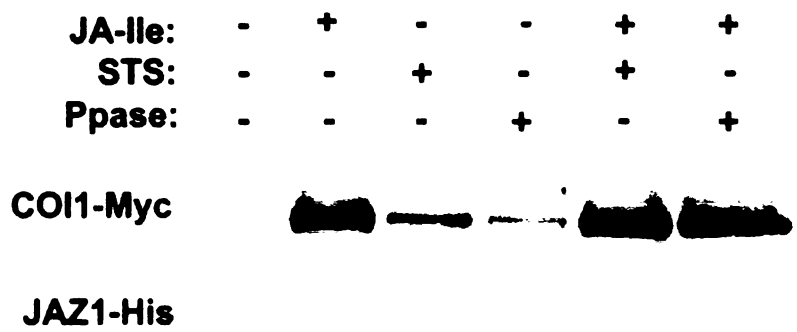

Figure 2.8. Effect of kinase and phosphatase inhibitors on JA-Ile-stimulated interaction between COI1-Myc and JAZ1-His.

Pull-down assays containing purified JAZ1-His and protein extract from *35S-COI1-Myc* plants were incubated in the presence (+) or absence (-) of 1 μM JA-Ile, 10 μM staurosporine (STS), and 5X Phosphatase Inhibitor Cocktail (PPase) as indicated. Assays were processed as described in the legend to Figure 5.

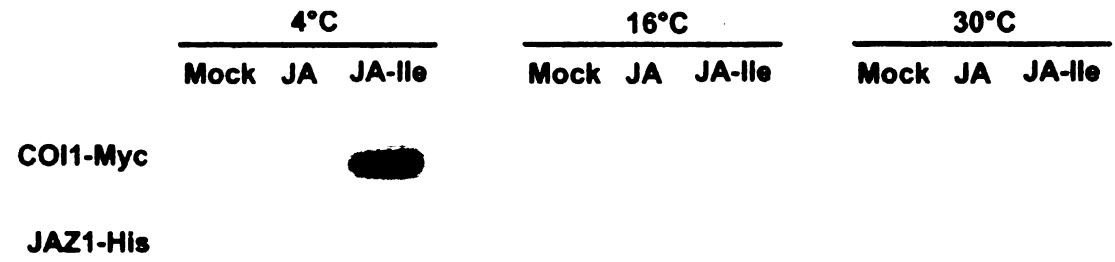

Figure 2.9. Effect of temperature on JA-Ile-mediated COI1-JAZ1 binding.

Pull-down reactions containing purified JAZ1-His and protein extract from *35S-COI1-Myc* plants were supplemented with 1 µM JA, 1 µM JA-Ile, or binding buffer (Mock). Reactions were incubated for 30 min at the indicated temperature and then incubated an additional 15 min at 4°C with 80 µL Ni resin, with gentle rocking. JAZ1-His complexes were recovered and assayed for COI1- Myc by immunoblotting. The Coomassie Blue-stained blot shows the recovery of JAZ-His by the Ni-affinity resin.

In pull downs conducted with such enzyme inhibitors, we would expect to see a negative effect on COI1-Myc recovery in the presence of JA-Ile if the addition or removal of a phosphate moiety was involved in complex formation. Neither staurosporine nor the phosphatase inhibitor cocktail had a significant effect on the interaction of COI1 with JAZ1 (Fig. 2.8). This result suggests that phosphoryl-transfer is not a requirement for JA-Ile promotion of a COI1-JAZ complex.

The original pull-down assays to detect the interaction of COI1 with JAZ was conducted at 4°C. To investigate the effect of temperature on the COI1-JAZ interaction, pull-down assays performed in the presence of JA or JA-Ile were incubated at 4, 16, and 30°C and were evaluated for the recovery of COI1-Myc (Fig. 2.9). The results showed that as the incubation temperature of the assays is increased to 16°C, the recovery of COI1-Myc by JAZ-His is abolished in JA-Ile-containing reactions. That higher temperatures failed to stimulate COI1-Myc recovery argues against the possibility that enzymatic activity is required for JA-Ile-mediated COI1-JAZ1 interaction. The loss of COI1-JAZ1 interaction at higher temperatures may results from the instability of any of the components in this assay. JA failed to promote COI1-Myc at any temperature.

To determine whether the JA-Ile dependent interaction of COI1-JAZ observed *in vitro* required accessory plant proteins, the interaction was tested in the yeast two-hybrid assay. JA-Ile included in the growth media stimulated a positive interaction between tomato COI1 and SlJAZ1 (Fig. 2.10). However, in the absence of JA-Ile or in the presence of JA, MeJA, and OPDA, no interaction was detected. This result demonstrates that no other plant proteins are required

to stimulate the COI1-JAZ interaction. Collectively, these results suggest that COI1-JAZ interaction does not require a JA-Ile-induced enzymatic modification of either protein, but rather that JA-Ile directly promotes the protein-protein interaction.

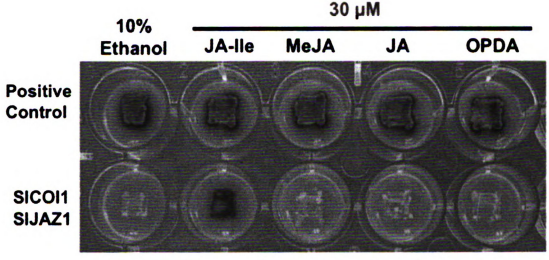

Figure 2.10. JA–Ile-dependent interaction between COI1 and JAZ1 in yeast.

Yeast two-hybrid protein-protein interaction assay. Dark colonies indicate a positive interaction light colonies indicate no interaction. Yeast growth media was supplemented with either ethanol, JA-Ile, MeJA, JA, or OPDA at the concentrations indicated. Colonies of a positive-control strain in, pLexA-53/pB42AD-Z, are shown (top row). Figure modified from Thines et al. 2007.

Discussion

The identification of JAZ proteins as substrates of COI1 is a major advance in our understanding of JA signaling. It also sets the stage to investigate how JA mediates COI1 dependent turnover of JAZ. We sought to address this question using a tomato line that expresses an epitope-tagged SlCOI1, which could be used to test COI1 recovery in JAZ-pulldown assays. In order to establish the approach with heterologous components, we identified seven *JAZ* genes in the tomato EST database. Wounding induced all tomato *JAZ* genes tested. The intensities and the timing of the expression among the different genes varied. *SlJAZ1*, *SGN-U327035*, and SGN-U315857 were the most rapidly and strongly induced, whereas SGN-U319732 and SGN-U320368 were very weakly induced. The *jai1-1* mutation, which abolishes COI1 function, eliminated *JAZ* transcript accumulation in response to wounding. Interestingly, basal levels of *JAZ3* transcript in *jai1-1* plants were decreased, indicating that constitutive expression of this gene is also regulated by COI1. The COI1-dependent response of *JAZ* genes in tomato is similar to the rapid accumulation of Arabidopsis *JAZ* transcripts in response to JA and wounding observed in (Chung et al. 2008, Thines et al. 2007). The rapid induction of these genes in tomato fits the hypothesis of Thines et al. that *JAZ* genes are induced quickly to negatively regulate their own synthesis.

In our efforts to study the biochemical requirements of COI1-mediated JAZ degradation, we developed an *in vitro* pull down assay to test for interactions between COI1 and JAZ. The creation of the *35S::COI1-Myc* line generated in the *jai1-1* background provides the advantage of assessing COI1-Myc-JAZ interaction in the absence of endogenous COI1. A significant challenge in establishing this assay was the expression and purification of a functional tomato JAZ protein. We showed that expressing JAZ in *E. coli* as an MBP/6xHis fusion yielded mg quantities of JAZ1-His protein that was ~90% pure. This method for expression and purification of JAZ in *E. coli* will likely be useful for purification and biochemical characterization of JAZ proteins from other plant species.

In taking advantage of this JAZ-His pull down assay, we extended the work of Thines et al. to demonstrate the basis of COI1-mediated JAZ degradation (Thines et al. 2007). The assay was used to demonstrate that JA-Ile mediates physical interaction between COI1 and JAZ1. We have determined by this assay that the specificity of the interaction of SlJAZ1 and COI1 is facilitated by JA-Ile and by JA-Leu to a lesser extent. That JA, MeJA, and OPDA do not promote this interaction indicates it is highly specific for JA- amino acid conjugate such as JA-Ile. Interestingly, the hydrophobic character of the JA amino acid conjugate is not sufficient for binding in pull down reactions containing JA-Trp or JA-Phe. The weak activity of JA-Leu is not surprising considering the similarity of its structure to JA-Ile.

Despite reports on the activity of jasmonic acid, MeJA, and OPDA, none of these molecules could promote COI1-JAZ1 binding (Browse 2005, Cheong et al.

2003, Stintzi et al. 2001). The characterization of the *jar1* mutant, which is unable to respond to JA or Me-JA due to a block in JA conjugation to Ile, provides the first evidence for a role for JA-Ile as an active ligand (Staswick et al. 2004). However, a role for other JAs cannot be excluded at this time as the *jar1* mutant still retains some JA responses, including male fertility that depends on COI1. A model in which one of the various JAs could promote the interaction of an as yet characterized JAZ protein with COI1, is one such scenario which could explain the residual JA responses of *jar1*. Alternatively, additional JA amino acid conjugating enzymes could be acting redundantly with JAR1 to produce JA-Ile. The later is supported by the detection of residual JA-Ile in *jar1* plants as well as the large group of GH3 type enzymes, like JAR1 present, in Arabidopsis (Chung et al 2008, Suza et al. 2008).

In the absence of a methodology to directly test JA-Ile binding to a COI1-JAZ complex, we exploited a pull down assay to characterize the conditions required for binding to occur. We found the requirements for COI1-JAZ binding resembled those described for the auxin induced TIR1-Aux/IAA interaction (Dharmasiri et al. 2003). Like TIR1, it appears that COI1-JAZ binding occurs independently of membrane or membrane associated proteins. Although several studies report roles for phospho-relay in modulating the JA response (Rojo et al. 1998, Kandoth et al. 2007), we were not able to detect a requirement for phosphoryl transfer in COI1-JAZ interactions. Our results do not exclude the possibility that COI1-JAZ can be modulated by phosphorylation. Indeed, a tobacco JAZ homolog was identified as a target of phosphorylation by an elicitor-

responsive mitogen-activated protein kinase (Katou et al. 2005). We addressed the requirement for of enzymatic activity in the tomato protein homogenate in contributing to JA-Ile-induced binding by testing COI1-JAZ interaction at various temperatures above 4°C. The loss of COI1-JAZ binding at elevated temperature indicates that the stability of some element of these pull-downs assays is jeopardized at higher temperatures. We can conclude that binding is not affected by an unknown enzymatic component. Yeast-two hybrid assays confirm the JA-Ile dependence of the COI1-JAZ interaction and demonstrate this interaction can occur at 30°C in the absence of additional plant proteins. Thus, the absence of *in vitro* COI1-JAZ binding at elevated temperatures is likely an artifact of the cell-free assay.

Collectively these results indicate that a COI1-JAZ complex is the site of JA-Ile perception, much like TIR1-Aux/IAA is the site of auxin perception. The approaches used here can be applied to COI1 and JAZ proteins from any species. By assessing the ligand dependence of a broad range of JAZs, the paradigm of JA-Ile-mediated COI1-JAZ binding can be fully evaluated. This research sets the stage for a better understanding of how the diverse array of JA responses are controlled by the action of COI1. Furthermore, the results described here will be instrumental to identify a jasmonate receptor.

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Chapter 3

COI1 is a critical component of a receptor for jasmonate and the bacterial virulence factor coronatine[2]

[2] This work has been published in Katsir, L., Schilmiller, A.L., Staswick, P.E., He, S.Y., & Howe, G.A. (2008) COI1 is a critical component of a receptor for jasmonate and the bacterial virulence factor coronatine. *Proc. Natl. Acad. Sci. U.S.A., 105*: 7100-5.

Abstract

Jasmonate is a lipid-derived hormone that regulates diverse aspects of plant immunity and development. An amino acid-conjugated form of jasmonate, jasmonoyl-isoleucine (JA-Ile), stimulates binding of the F-box protein CORONATINE INSENSITIVE1 (COI1) to, and subsequent ubiquitin-dependent degradation of, JAsmonate ZIM-domain (JAZ) proteins that repress transcription of jasmonate-responsive genes. The virulence factor coronatine (COR), which is produced by several plant pathogenic strains of *Pseudomonas syringae*, suppresses host defense responses by activating jasmonate signaling in a COI1-dependent manner. Although previous data indicate that COR acts as a molecular mimic of JA-Ile, the mechanism by which JA-Ile and COR are perceived by plant cells remains unknown. Here we show that interaction of tomato COI1 with divergent members of the JAZ family is highly specific for JA-Ile and structurally related JA conjugates, and that COR is at least 100-fold more active than JA-Ile in promoting this interaction *in vitro*. JA-Ile competes for binding of COR to COI1-JAZ complexes, demonstrating that COR and JA-Ile are recognized by the same receptor. Binding of COR to the COI1-JAZ complex requires COI1 and is severely impaired by a point mutation in the putative ligand-binding pocket of COI1. Finally, we show that the highly conserved C-terminal domain of JAZ3 is necessary and sufficient for ligand-induced COI1-JAZ interaction and ligand binding. These findings demonstrate that COI1 is a critical component of the jasmonate receptor and that pathogens can manipulate hormone receptors in their hosts to establish infection.

Introduction

Jasmonic acid (JA) and its bioactive derivatives, collectively referred to as jasmonates (JAs), regulate a wide range of physiological processes in higher plants. JAs have a well-established role in orchestrating genome-wide transcriptional changes in response to biotic stress and developmental cues (Blabi et al. 2008, Howe et al. 2008, Browse et al. 2008, Wasternack et al. 2007). In general, JAs promote defensive and reproductive processes, as well as inhibit the growth and photosynthetic output of vegetative tissues. These juxtaposing activities suggest a general role for the hormone in controlling resource allocation between growth- and defense-related processes, thus optimizing plant fitness in rapidly changing and hostile environments.

Recent studies indicate that JA controls the expression of early response genes by promoting the ubiquitin-dependent degradation of jasmonate ZIM domain (JAZ) proteins (Thines et al. 2007, Chini et al. 2007, Yan et al. 2007). The JAZ family of proteins in *Arabidopsis* consists of 12 members, which have been classified as a subgroup of the larger family of TIFY proteins that share a conserved TIF[F/Y]XG motif within the ZIM domain (Vanholme et al. 2007). A second defining feature of JAZs is the highly conserved Jas motif, which has a $SLX_2FX_2KRX_2RX_5PY$ consensus sequence near the C terminus (Thines et al. 2007, Chini et al. 2007, Yan et al. 2007). Genetic analysis indicates that coronatine-insensitive 1 (COI1), an F-box protein that determines the target specificity of the E3 ubiquitin ligase SCF^{COI1} (where SCF indicates Skp/Cullin/F-box), is required for most, if not all, JA-signaled processes (Feys et al. 1994, Xie

et al. 1998, Li et al. 2004). *Arabidopsis* JAZ3 (also known as JAI3) interacts with and presumably represses the activity of the MYC2 transcription factor that promotes the expression of JA-responsive genes (Chini et al. 2007). Current models indicate that degradation of JAZ repressors by the SCF^{COI1}–ubiquitin–proteasome pathway in response to a bioactive JA signal relieves the inhibition of MYC2, thereby activating the expression of early response genes. Functional analysis of the tomato homologs of COI1, JAZ, and MYC2 (Thines et al. 2007, Li et al. 2004, Boter et al. 2004) indicates that this general mechanism of JA signaling is conserved in the plant kingdom.

The *JAR1* gene encodes a JA–amido synthetase that catalyzes the formation of jasmonoyl-L-isoleucine (JA–Ile) (Staswick et al. 2002, Staswick et al. 2004, Suza et al. 2008). The JA-insensitive phenotype of *jar1* mutant plants indicates that JA–Ile is an active signal in the JA pathway. Analysis of *jar* mutants also showed that JA–Ile is important for the regulation of plant defense responses to attack by pathogens and insects (Staswick et al. 2004, Staswick et al. 1998, Kang et al. 2006). Thines *et al.* (Thines et al. 2007) recently showed that formation of both *Arabidopsis* and tomato COI1–JAZ1 complexes is stimulated by JA–Ile but not by jasmonic acid, methyl-JA (MeJA), or the JA precursor 12-oxo-phytodienoic acid (OPDA). Endogenous JA–Ile levels increase within minutes of tissue damage, coincident with the expression of early JA-response genes (Chung et al. 2008). It is not known whether the JA–Ile-dependent interaction with COI1 is unique to JAZ1 or is more generally applicable to other members of the JAZ family.

The mechanism by which JA–Ile promotes COI1 binding to JAZ proteins remains to be determined. In yeast and animal cells, target recognition by E3 ubiquitin ligases typically depends on phosphorylation or other posttranslational modifications of the substrate (Deshaies et al. 1999). Notably, COI1 is homologous to TIR1, which functions as a receptor for the plant hormone auxin (Tan et al. 2007). Auxin regulates gene expression by binding to TIR1 and stimulating the ubiquitin–proteasome-dependent degradation of Aux/IAA transcriptional repressors (Dharmasiri et al. 2005, Kepinski et al. 2005). Despite the many similarities between auxin and JA signaling, the identity of the JA receptor and its physiological ligand(s) is not known.

Coronatine (COR) is a phytotoxin produced by some plant pathogenic strains of *Pseudomonas syringae* (Bender et al. 1999). Several lines of evidence indicate that COR exerts its virulence effects by activating the host's JA signaling pathway (Feys et al. 1994, Zhao et al. 2003, Lauchli et al. 2003, Uppalapati et al. 2005, Thilmony et al. 2006). The insensitivity of *coi1* mutants of *Arabidopsis* and tomato to COR demonstrated that COI1 is required for the action of the toxin (Feys et al. 1994, Zhao et al. 2003). These observations, together with the structural similarity of COR to JA–Ile, support the notion that this virulence factor acts as a molecular mimic of JA–Ile (Staswick et al. 2004, Krumm et al. 1995).

To test directly the hypothesis that JA–Ile and COR share a common molecular mechanism of action, we used an *in vitro* pull-down assay to assess the ability of COR, JA–Ile, and structurally related JA conjugates to promote the interaction of tomato COI1 with two divergent tomato JAZ proteins. Here, we

provide evidence that COI1, or possibly a COI1–JAZ complex, is a receptor for JA–Ile and that COR exerts its virulence effects by functioning as a potent agonist of this receptor system. We also show that the Jas motif-containing C-terminal region of JAZ3 is necessary and sufficient for hormone-induced interaction of COI1 with JAZ3.

Materials and methods

Biological materials

Growth conditions for *Solanum lycopersicum* (tomato) were described previously (Li et al. 2001). A *35S-COI1–Myc* transgenic line of tomato (cv Microtom) was used as a source of COI1–Myc in pull-down experiments (Thines et al. 2007). The tomato *jai1-1* and *jai1-3* (previously called *spr5*) mutants (cv Castlemart) were isolated and propagated as described. (Li et al. 2004 and Li et al. 2001).

Chemicals

Coronatine (C8115), phosphatase inhibitor mixture 1 (P2850), staurosporine (S4400), indole-3-acetic acid (I-2886), salicylic acid (S7401), and (±)-JA were purchased from Sigma. Jasmonoyl–amino acid conjugates were prepared, and the structures were verified by GC-MS as described previously (Staswick et al. 2004, Kramell et al. 1988). The naturally occurring (–)-JA–Ile isomer was separated from (+)-JA–Ile by HPLC. (–)-JA–Ile was used in pull-down assays, whereas all other JA conjugates were used as a mixture of the (+)-JA– and (–)-JA–amino acid diastereomers. [^{3}H]COR was prepared commercially (GE Healthcare) by using hydrogen–tritium exchange between tritiated water and unlabeled COR. The specific activity of [^{3}H]COR as determined by mass spectrometry is 333 GBq per mmol. The radiochemical purity was 97.0% as determined by HPLC.

Cloning and expression of JAZ fusion proteins

A full-length cDNA for *SlJAZ3* was PCR-amplified from a tomato EST clone (EST555543; BI935654) provided by the SOL Genomics Network (Cornell University). Primers used for the PCR were 5'-GCGCGGCCGCCGAGATGGAGAGGGACTTTATGG-3' and 5'-ACGCGTCGACTAGCTTGGTCTCCTTACCG-3'. The resulting PCR product was cleaved with NotI and SalI and cloned into the corresponding restriction sites of pRMG-nMAL (Thines et al. 2007) to make the MBP–JAZ3–His_6 fusion plasmid. Truncated derivatives of JAZ3, designated $JAZ3_{1-221}$ and $JAZ3_{149-306}$, were amplified by using the following two primer sets, respectively: 5'-GCGCGGCCGCCGAGATGGAGAGGGACTTTATGG-3' and 5'-CCCTCGAGCACATTAGGTGGAGCCA-3'; 5'-GCGCGGCCGCCTGTGCTCCACCTAAT-3' and 5'-CCTCGAGGGTCTCCTTACCGGCTAA-3'. The PCR products were cleaved with NotI and XhoI and cloned into the corresponding sites of pRMG-nMAL. Full-length JAZ3 and JAZ1 fusion proteins, as well as the $JAZ3_{1-221}$ and $JAZ3_{149-306}$ truncations, were expressed in *Escherichia coli* and purified by Ni affinity chromatography as described (Thines et al. 2007).

Pull-down and [^{3}H]COR binding assays

A *35S-COI1–Myc* transgenic line of tomato was used as a source of COI1–Myc in a JAZ–His pull-down assay described previously (Thines et al. 2007). Unless otherwise indicated, the quantity of JAZ–His added to each reaction was 25 μg.

Protein concentrations were determined with a BCA protein assay kit (Pierce). Standard [^{3}H]COR-binding assays contained 50 μg of purified JAZ–His protein, 4 mg of total leaf protein from *35S::COI1–Myc* plants or an otherwise indicated genotype, and 400 nM [^{3}H]COR (1.86 μCi) in a final volume of 0.5 ml of binding buffer [50 mM Tris, pH 6.8/10% glycerol, 100 mM NaCl, 25 mM imidazole, 20 mM 2-mercapto-ethanol, 10 μM MG132, 0.1% Tween 20, and Complete Mini protease inhibitor tablet-EDTA free (Roche)] and were performed in triplicate. Reactions were incubated at 4°C for 30 min, after which 80 μl of Ni resin was added. After an additional 15-min incubation at 4°C, JAZ–His-bound Ni resin was washed three times on microcentrifuge spin columns with 0.25 ml of binding buffer at 4°C. JAZ–His was eluted from the resin with 100 μl of 300 mM imidazole. Radioactivity in the resulting eluent was measured by scintillation counting (MicroBeta Trilux; PerkinElmer) after the addition of 1 ml of scintillation fluid (Optiphase Supermix; PerkinElmer).

Saturation-binding experiments were performed with increasing concentrations of [^{3}H]COR in the presence or absence of 100-fold excess unlabeled COR. For experiments by using *jai1-1* extracts, pull-down reactions contained 5 mg of total leaf protein, 50 μg of JAZ3–His, and 400 nM [^{3}H]COR with or without the addition of 400 μM unlabeled COR. Saturation-binding experiments comparing WT and *jai1-3* leaf extracts were conducted by incubating 5 mg of leaf protein and 50 μg of JAZ3–His with increasing concentrations of [^{3}H]COR (10, 100, 500, and 1,000 nM) in the presence or absence of 100-fold excess unlabeled COR.

Analysis of Proteinase Inhibitor II Expression. Proteinase inhibitor II (PI-II) protein and mRNA levels were measured according to published procedures Li et al 2004, Li et al. 2001). Probed RNA blots were visualized with a phosphorimaging device, and the signal intensities quantified with the Quantity One-4.2.2 program (Bio-Rad). Values for each time point were normalized to the *eIF4A* loading control.

Results

Requirement of jasmonoyl–amino acid conjugates for COI1 interaction with two divergent JAZs

We previously used an *in vitro* pull-down assay to demonstrate that JA–Ile stimulates interaction between tomato COI1 and a purified JAZ1–His fusion protein in the absence of intact cells (Thines et al. 2007). Recovery of COI1–Myc by JAZ1–His was not promoted by jasmonic acid (at concentrations up to 1 mM; data not shown), MeJA, or the JA precursor OPDA (Thines et al. 2007). To further define the specificity of JA–Ile as a signal for COI1–JAZ1 interaction, we compared the activity JA–Ile with other naturally occurring jasmonoyl–amino acid conjugates. JA–amino acid conjugates containing small hydrophobic amino acids stimulated COI1–JAZ1 binding to varying degrees, with the relative order of activity being JA–Ile, → JA–Val, → JA–Leu (Figure 3.1A). JA–Ala was very weakly active, whereas JA–Phe and JA–Gln were inactive at the highest concentration tested (10 μM).

To determine whether the signal specificity of JAZ1 extends to other members of the tomato JAZ family, we identified a tomato *JAZ* cDNA whose sequence is significantly diverged from *JAZ1*. BLAST and phylogenetic analyses indicated that the tomato protein encoded by this cDNA is most similar to *Arabidopsis* JAZ3 (Figure 3.2). We therefore refer to this tomato protein as *Sl*JAZ3 (or hereafter simply as JAZ3).

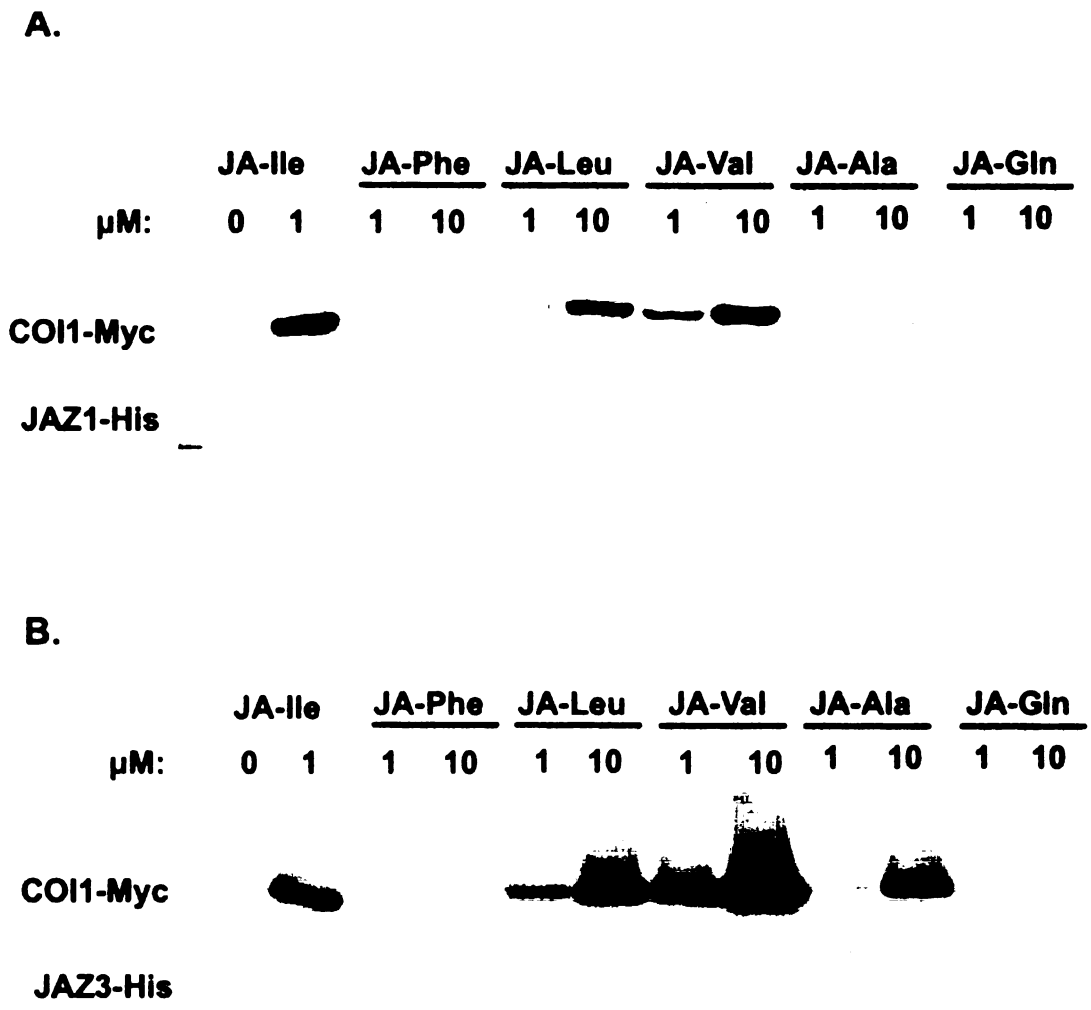

Figure 3.1. Specificity of JA–amino acid conjugates in promoting COI1–JAZ interaction. Pull-down assays were performed with recombinant JAZ1–His A, or JAZ3–His B, and extracts from 35S-COI1–Myc plants. Assays were supplemented with various JA–amino acid conjugates at the indicated concentration and incubated for 30 min at 4°C. Protein bound to JAZ1–His or JAZ3–His was analyzed by immunoblotting for the presence of COI1–Myc. The Coomassie Blue-stained blot in each panel shows the recovery of JAZ–His by the Ni affinity resin.

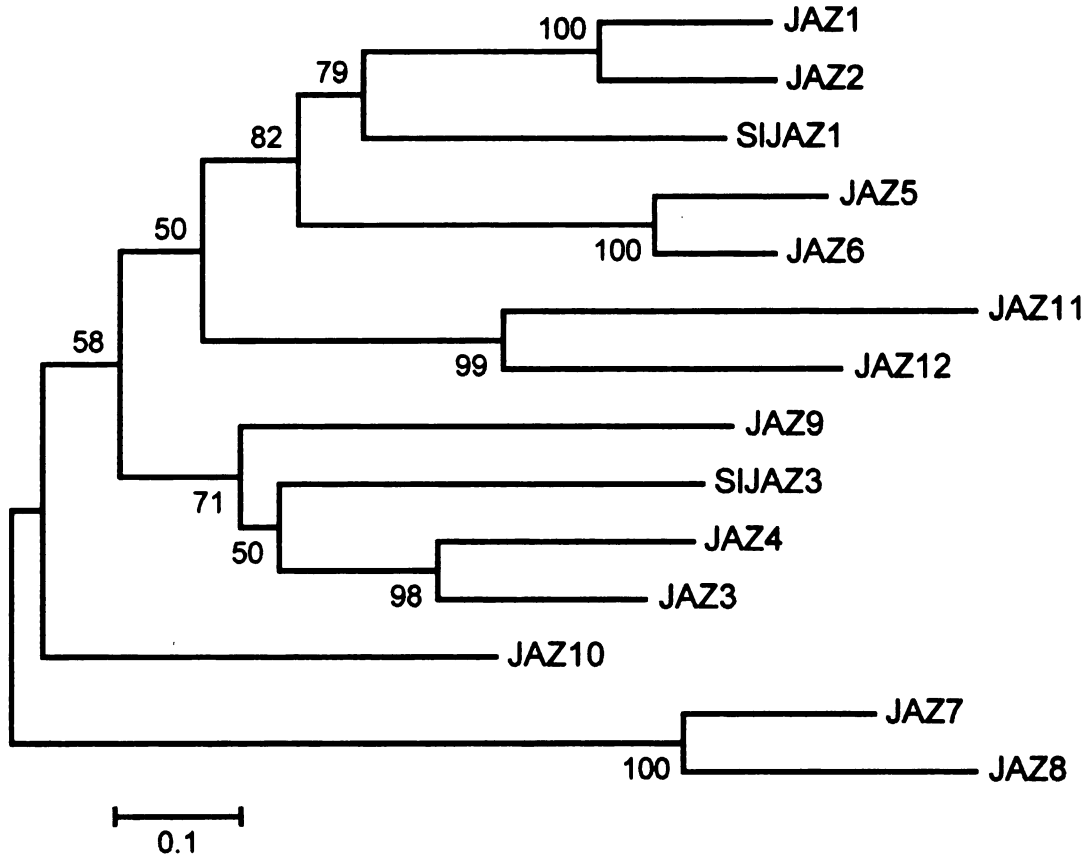

Figure 3.2. Phylogenetic tree showing the relationship of tomato JAZ1 (SlJAZ1) and JAZ3 (SlJAZ3) to the 12 JAZ proteins in *Arabidopsis thaliana*.

BLAST analysis indicated that tomato JAZ3 (SlJAZ3) is most similar to Arabidopsis JAZ3/JAI3. The predicted amino acid sequence of SlJAZ3 exhibits hallmark features of the JAZ protein family, including the highly conserved ZIM and Jas motifs. The phylogenetic tree was constructed with the neighbor-joining method by using MEGA 4 software (Molecular Evolutionary Genetics Analysis). The value on each node is the percentage of the bootstrap value (only values 50 are shown).

The sequence similarity between tomato JAZ1 and JAZ3 (27% amino acid identity) is restricted mainly to the ZIM domain and C-terminal Jas motif.

Pull-down assays performed with a JAZ3–His fusion protein showed that the chemical specificity for COI1–Myc binding to JAZ3–His is very similar to that for JAZ1–His. For instance, the COI1–JAZ3 interaction was strongly promoted by JA–Ile, whereas no stimulatory effect was observed with jasmonic acid, MeJA, OPDA, JA–Phe, or JA–Gln (Figure 3.1B and data not shown). JA–Leu, –Val, and –Ala stimulated recovery of COI1–Myc by JAZ3–His to various extents, with the relative order of activity being JA–Ile → JA–Val → JA–Leu → JA–Ala. JA–Val and JA–Ala were more effective in stimulating COI1–Myc binding to JAZ3–His than to JAZ1–His, suggesting that formation of COI1–JAZ1 complexes *in vitro* may be more selective for JA–Ile than for COI1–JAZ3 complexes. We conclude that recruitment of two divergent JAZ proteins by tomato COI1 is promoted specifically by JA–Ile and structurally related JA conjugates.

Coronatine Binds Directly to COI1–JAZ Complexes. The ability of the *P. syringae* toxin COR to activate JA signaling in a COI1-dependent manner (Feys et al. 1994, Zhao et al. 2004, Uppalapati et al. 2005, Thilmony et al. 2006) together with the structural similarities between COR and JA–Ile (Figure 3.3A), led us to hypothesize that COR exerts its virulence effects by promoting COI1–JAZ interactions.

Figure 3.3. Coronatine promotes formation of COI1–JAZ complexes.

A, Molecular structures of JA–Ile and COR. B, JAZ1–His-containing pull-down assays supplemented with buffer (indicated by 0) or various concentrations of COR were processed as described in the legend to Figure 3.1. C, Comparison of the activity of JA–Ile and COR in promoting COI1 interaction with JAZ1. Pull-down assays containing purified JAZ1–His and protein extract from *35S–COI1–Myc* plants were incubated in the absence (indicated by 0) or presence of the indicated concentration of JA–Ile or COR. The Coomassie blue-stained blot shows recovery of JAZ1–His.

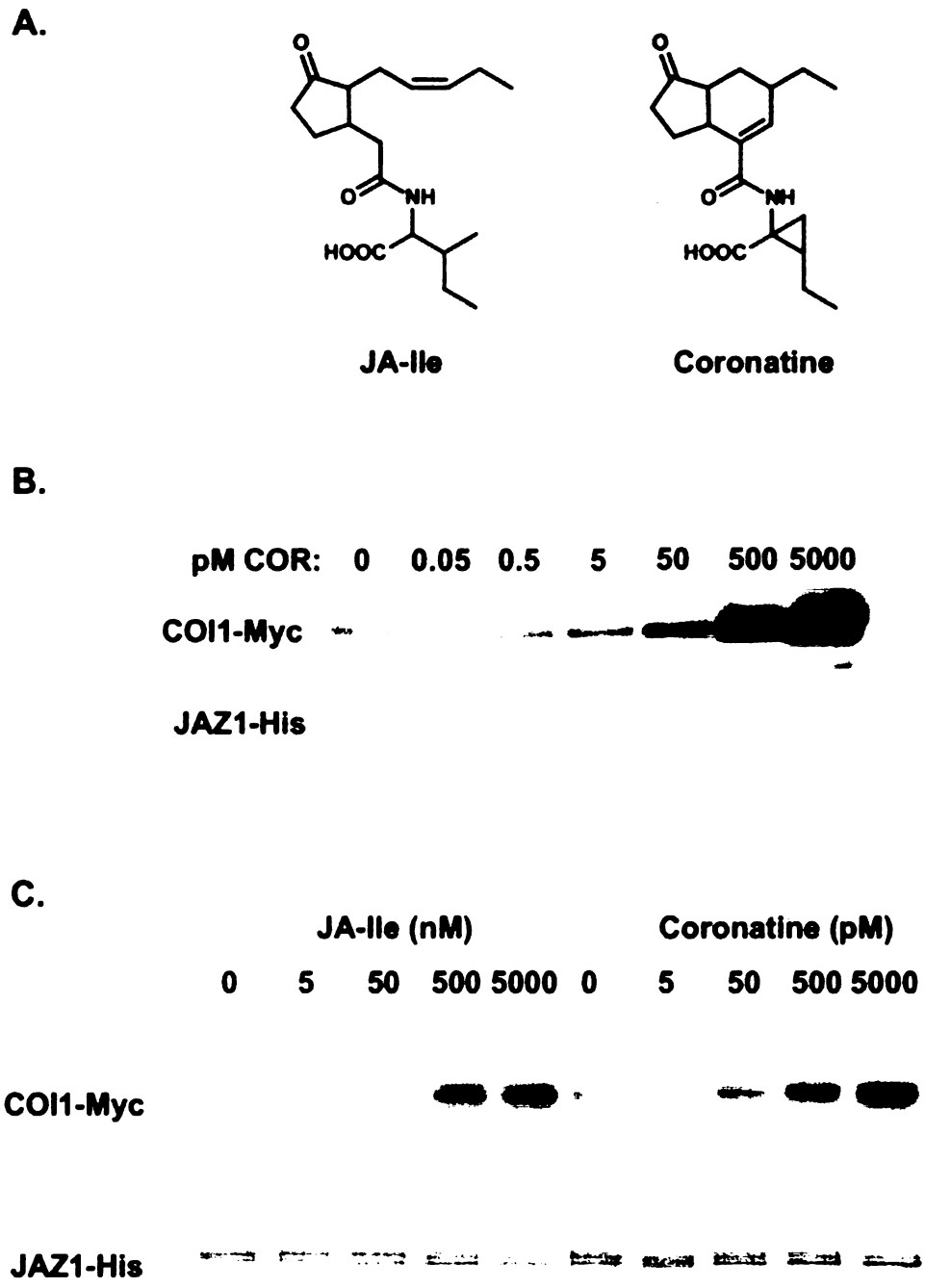

Figure 3.3. Coronatine promotes formation of COI1–JAZ complexes.

We found that COR promotes COI1–Myc binding to JAZ1–His in a dose-dependent manner and, remarkably, that this stimulatory effect was apparent at

concentrations of COR as low as 50 pM (Figure 3.3B). Direct comparison of the activity of COR with that of JA–Ile showed that the toxin is 1,000-fold more active than JA–Ile (Figure 3.3C), which is in agreement with the previous observation that 50–500 nM concentrations of JA–Ile are required to stimulate COI1–JAZ1 interaction in this assay (Thines et al. 2007). COR was at least 100-fold more effective than JA–Ile in promoting the COI1–JAZ3 interaction in the JAZ3–His pull-down assay (data not shown).

To determine whether COR is a ligand that directly binds to the COI1–JAZ complex, we performed binding assays in which crude leaf extracts containing COI1–Myc were incubated with [^{3}H]COR and either JAZ1–His or JAZ3–His. The results in Figure 3.4A show that both JAZ1–His and JAZ3–His retain [^{3}H]COR after isolation of the fusion protein by Ni-affinity chromatography and subsequent washing steps. The ability of unlabeled COR to compete with [^{3}H]COR for binding indicates that the binding is specific. Binding of [^{3}H]COR to JAZ3–His complexes was competed by the addition of 10,000-fold excess unlabeled JA–Ile but not by the addition of the same amount of jasmonic acid (Figure 3.4B), indicating that the COR receptor eluting with JAZ3–His also binds to JA–Ile. Similar results were obtained in pull-down assays performed with JAZ1–His (Figure 3.4C). Scatchard analysis of saturation binding data obtained with the JAZ3–His pull-down assay showed that the apparent dissociation constant (K_d) of the receptor for COR was 20 nM (Figure 3.4D).

Figure 3.4. Coronatine and JA-Ile bind to a COI1-JAZ complex.

A, Pull-down reactions containing extracts from *35S-COI1–Myc* plants and JAZ3–His (filled circles) or JAZ1–His (open circles) were incubated with [^{3}H]COR in the presence of increasing concentrations of unlabeled COR. Radioactivity recovered with JAZ–His is indicated (CPM). B, Pull-down reactions containing *35S-COI1–Myc* leaf extract, JAZ3–His, and [^{3}H]COR were incubated in the absence (indicated by 0) or presence of the indicated amount of unlabeled COR, JA (jasmonic acid), or JA–Ile. C, Specific binding of COR to a COI1–JAZ1 protein complex. Pull-down reactions containing *35S–COI1–Myc* leaf extract, JAZ1–His, and [^{3}H]COR were incubated in the absence (indicated by 0) or presence of the indicated amount of unlabeled COR, JA (i.e., jasmonic acid), or JA–Ile. Radioactivity recovered with JAZ1–His is indicated (in cpm). Error bars denote the SD of triplicate assays. D, Pull-down reactions containing *35S-COI1–Myc* leaf extract, JAZ3–His, and increasing concentrations of [^{3}H] COR were used to construct a saturation curve for specific binding. Shown is a Scatchard plot of the saturation-binding data for a representative experiment. Error bars denote the SD of triplicate assays.

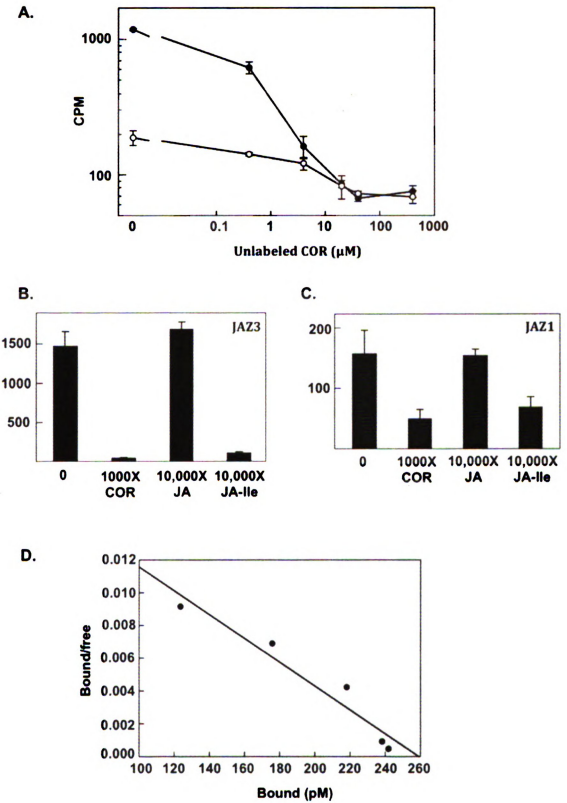

Figure 3.4. Coronatine and JA-Ile bind to a COI1-JAZ complex.

Specific binding of COR to the JAZ3–His complex was observed in pull-down reactions supplemented with crude leaf extract from WT tomato leaves (Figure 3.5). Thus, like COI1–Myc, endogenous COI1 interacts with JAZ3–His in a COR-dependent manner.To determine whether COI1 is required for COR binding, pull-down reactions were performed with leaf extracts from the COI1-deficient *jai1-1* mutant of tomato that harbors a deletion in the *SlCOI1* gene (Li et al. 2004). As shown in Figure 3.5, *jai1-1* extracts failed to promote recovery of [^{3}H]COR by JAZ3–His. Binding assays performed with JAZ3–His in the absence of tomato leaf extract showed that COR does not bind specifically to JAZ3–His (Figure 3.5). We thus conclude that COI1 is an essential component of the COR/JA–Ile receptor and that JAZ protein alone is not sufficient for high-affinity ligand binding. Homology between COI1 and the TIR1 auxin receptor provides indirect evidence for the idea that COI1 is a JA receptor (Tan et al. 2007, Parry et al. 2006).

To define further the role of COI1 in COR binding, we used the *jai1-3* tomato mutant that harbors a point mutation (L418F) in the leucine-rich repeat domain of COI1 and, as a consequence, is partially insensitive to JA (Figure 3.6A, B). The tomato *jai1-3* mutant, formerly called *spr-5* (Li et al. 2001), was isolated in a genetic screen for mutants that are suppressed in systemin-induced defense responses (Li et al. 2001). Sequencing of two independent *SlCOI1* cDNAs from *jai1-3* plants revealed a single G to T base change at position 1,254 of the cDNA ORF. This change results in substitution of Leu-418 with a Phe.

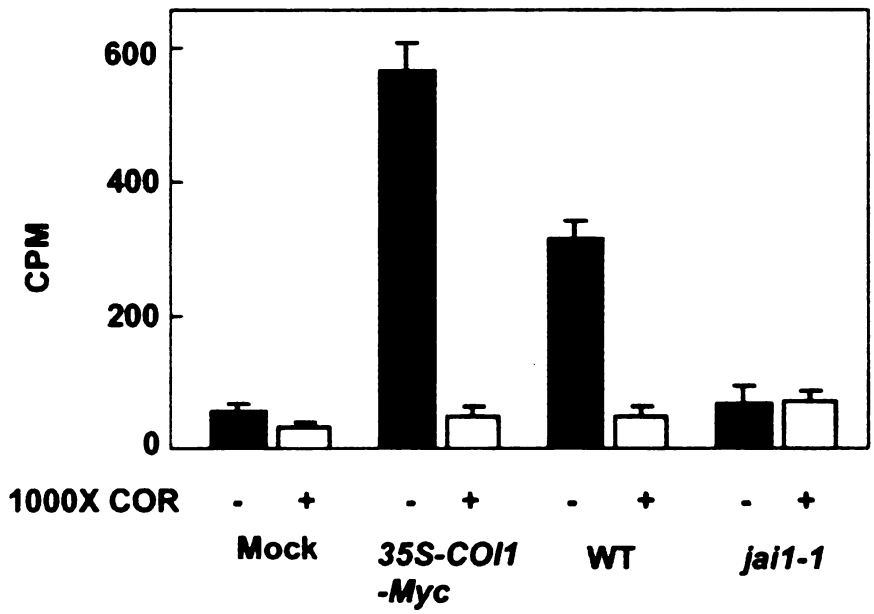

Figure 3.5. COI1 is an essential component of the jasmonate receptor.

Pull-down reactions containing JAZ3–His, [^{3}H]COR, and crude leaf extract from the indicated tomato genotype (or an equivalent volume of buffer; indicated by Mock) were incubated in the presence (+) or absence (−) of 1,000-fold unlabeled COR. The amount of radioactivity recovered in the JAZ3–His complex is shown. Error bars denote the SD of triplicate assays.

Northern blot analysis of the JA-inducible proteinase inhibitor II gene showed that *jai1-3* leaves are approximately 100-fold less responsive than WT leaves to exogenous MeJA (Figure 3.7A). The mutated Leu residue in *jai1-3* aligns with an Ile residue (Ile-406) in TIR1 that contacts the Aux/IAA peptide substrate within the auxin-binding pocket of TIR1 (Tan et al. 2007). We hypothesized that *jai1-3* might impair COI1 interaction with either the ligand or the JAZ substrate. As shown in Figure 3.7B, [^{3}H]COR was recovered by JAZ3–His in pull-down assays supplemented with *jai1-3* leaf extract but to a level that was much less than that obtained with WT extract. At 500 nM COR, for example, the recovery of specific binding in the presence of *jai1-3* extract was only 1.3-fold above background, which was 10-fold lower than the amount of specific binding recovered in the presence of WT extract. These results indicate that the reduced sensitivity of *jai1-3* leaves to exogenous JA correlates with reduced binding activity of *jai1-3* extract to COR and provide evidence that Leu-418 of COI1 plays a role in the formation of a stable complex between COI1, JAZ, and COR.

Figure 3.6. The tomato *jai1-3* mutant contains a Leu418Phe amino acid substitution in COI1 that results in reduced sensitivity to endogenous and exogenous JA.

A, Effect of *jai1-3* on MeJA-induced root growth inhibition. Wild-type, *jai1-1*, and *jai1-3* seedlings were treated with either water (-) or 1 mM MeJA (+) as previously described (Li et al. 2004). Seedlings were photographed 9 days after seed sowing. Note that the root length of MeJA-treated *jai1-3* seedlings is intermediate between that of WT and *jai1-1* seedlings. B, PI-II deficiency in *jai1-3* plants is restored by high concentrations of exogenous MeJA. Two-leaf-stage wild-type (filled bar) and *jai1-3* (open bar) tomato plants were mechanically wounded on each leaf with a hemostat (wounded leaf). Four additional sets of plants were exposed to the indicated amount (in μl) of pure MeJA or a mock control (indicated by 0) in an enclosed container (MeJA-treated leaf) as described (Li et al. 2004). PI-II protein levels in the leaf tissue were measured 1 day after treatment. PI-II levels were also measured in wild-type and *jai1-3* flowers (flwr). Data points represent the mean SD of at least six plants per treatment group.

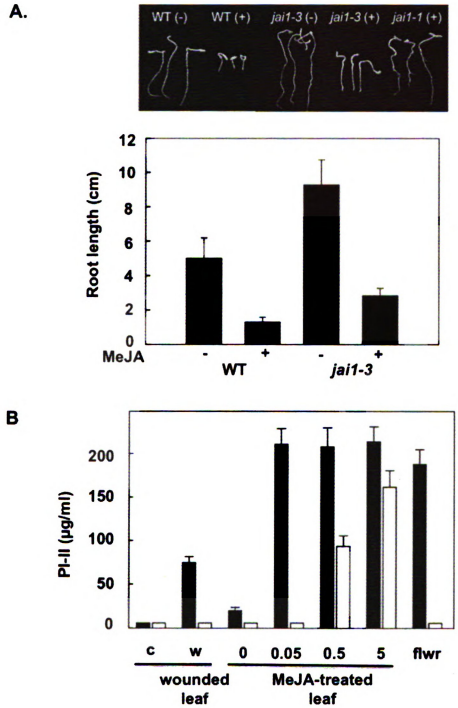
A.
WT (-)
WT (+)
jai1-3 (-)
jai1-3 (+)
jai1-1 (+)
Root length (cm)
12
10
8
6
4
2
0
MeJA
-
+
-
+
WT
jai1-3
B
PI-II (µg/ml)
200
150
100
50
0
c
w
0
0.05
0.5
5
flwr
wounded leaf
MeJA-treated leaf

Figure 3.6.

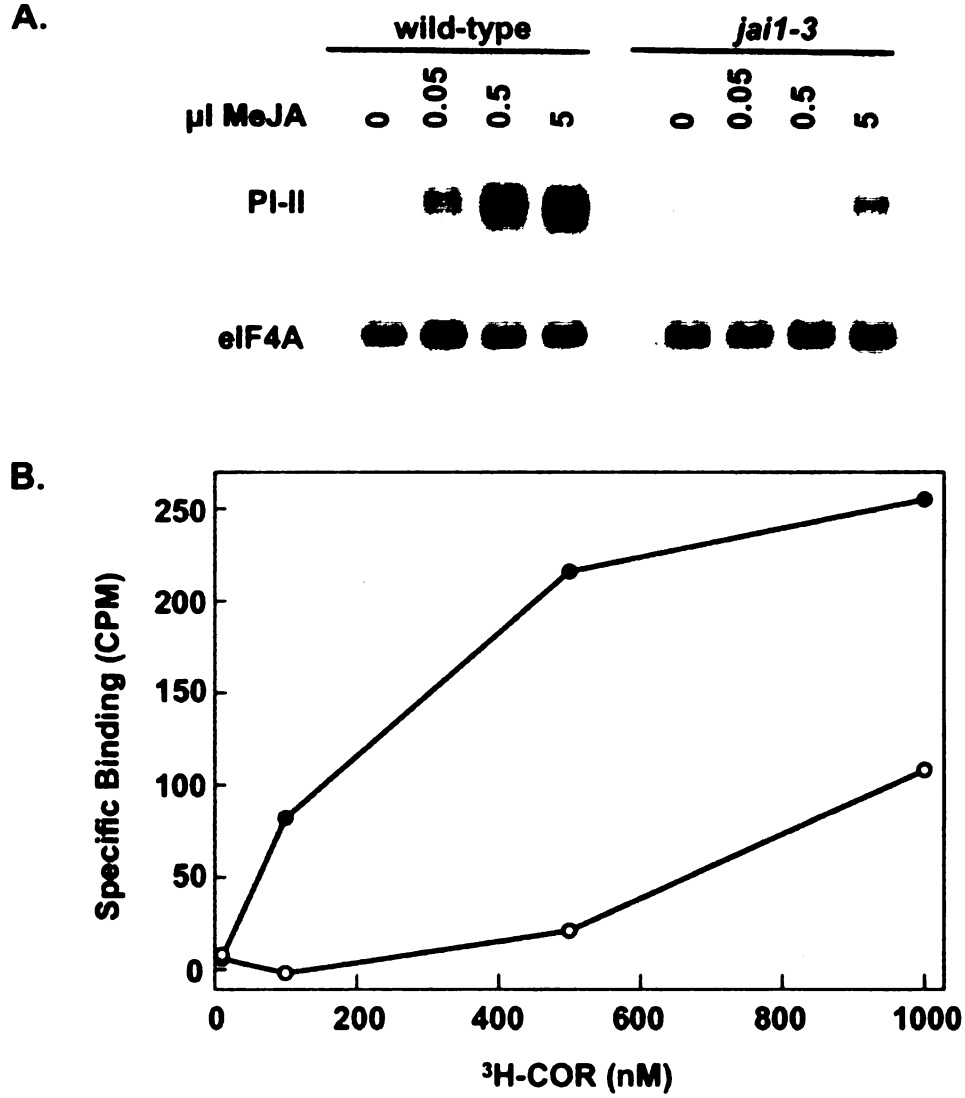

Figure 3.7. A Leu418Phe amino acid substitution in COI1 results in reduced sensitivity to exogenous JA and reduced affinity for COR

A, Northern blot analysis of proteinase inhibitor II transcript accumulation in wild-type and *jai1-3* plants treated in a closed container for 10 h with various amounts of vaporous MeJA. Blots were hybridized to an eIF4A cDNA as a loading control. B, Specific binding of [^{3}H]COR in pull-down assays containing JAZ3–His and leaf extract from either WT (closed circles) or *jai1-3* (open circles) plants.

The C-Terminal Region of JAZ3 Interacts with COI1 and Promotes Ligand Binding.

Having established that COR and JA–Ile bind to, and stimulate the formation of, a COI1–JAZ3 protein complex, we sought to identify the region of JAZ3 that facilitates this interaction. Truncated derivatives ($JAZ3_{1-221}$ and $JAZ3_{149-306}$) of JAZ3 containing either the conserved TIFYXG motif in the ZIM domain or the Jas motif (Figure 3.8A) were expressed as maltose-binding protein (MBP)–JAZ–His fusion proteins and tested for their ability to interact with COI1–Myc (Figure 3.8A). $JAZ3_{149-306}$–His efficiently recovered COI1–Myc in a JA–Ile-dependent manner, whereas $JAZ3_{1-221}$–His did not (Figure 3.8B). Moreover, we found that specific binding of [^{3}H]COR was recovered by $JAZ3_{149-306}$–His but not $JAZ3_{1-221}$–His in assays containing *35S-COI1*–Myc extract (Figure 3.8C). These results show that tomato $JAZ3_{149-306}$ is necessary and sufficient for the COI1–JAZ3 interaction and specific binding of COR to the COI1–JAZ3 complex.

Figure 3.8. The C-terminal region of JAZ3 is required for COI1 interaction and specific binding of COR to the COI1–JAZ3 complex. A, Schematic diagram of full-length and truncated JAZ3 constructs. The positions of the conserved ZIM (gray box) and Jas (black box) motifs are shown. The N-terminal MBP and C-terminal His_6 fusions are not shown. B, Pull-down assays containing 35S-COI1–Myc leaf extract and the indicated JAZ3 fusion protein were incubated in the presence (+) or absence (−) of 1 μM JA–Ile. Reactions were processed as described in the legend to Fig. 1. C, Pull-down reactions containing 35S-COI1–Myc leaf extract, [^{3}H]COR, and the indicated JAZ3 fusion protein were incubated in the presence (+) or absence (−) of unlabeled COR

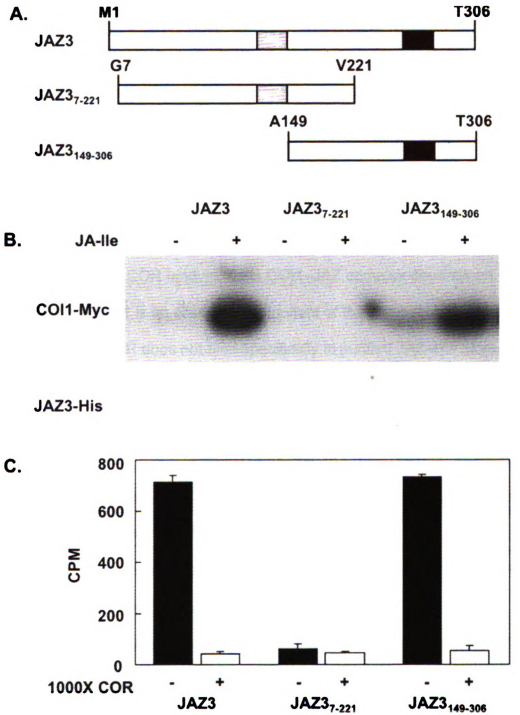
A.
M1
T306
JAZ3
G7
V221
JAZ3 7-221
A149
T306
JAZ3 149-306
JAZ3
JAZ3 7-221
JAZ3 149-306
B.
JA-Ile
- + - + - +
COI1-Myc
JAZ3-His
C.
800
600
400
200
0
CPM
1000X COR
- + - + - +
JAZ3
JAZ3 7-221
JAZ3 149-306

Figure 3.8.

Discussion

In this present study, we used a direct ligand-binding assay to investigate the mechanism by which COR and JA–Ile mediate the interaction between tomato COI1 and JAZ proteins. Our results indicate that JA–Ile does not act indirectly to induce an enzymatic modification of COI1 or JAZ but rather works directly to promote the COI1–JAZ interaction. The ability of JA–Ile to compete with COR for specific binding indicates that COR and JA–Ile are recognized by the same receptor. Because COR binding to the COI1–JAZ complex depends on COI1, we conclude that COI1 is an essential component of the perception apparatus. Moreover, that COR does not bind specifically to purified JAZ–His alone or in the presence of COI1-deficient tomato extract excludes the possibility that JAZ proteins function as JA receptors. These observations, together with the fact that JA–Ile-induced interaction between COI1 and JAZ does not require any other plant protein (Thines et al. 2007), provide strong evidence that COI1 is a receptor that specifically binds JA–Ile and COR.

Our results also provide evidence that the molecular mechanism of JA–Ile action is similar to that of auxin, which promotes substrate recruitment by creating a surface on the leucine-rich repeat domain of TIR1 that facilitates Aux–IAA binding (Tan et al. 2007). Detection of a stable ternary COI1–ligand–JAZ complex in the pull-down assay is consistent with the idea that JA–Ile/COR interact simultaneously with COI1 and JAZ. Because our binding assay relies on the recovery of components that copurify with JAZ–His, it remains to be determined

whether COI1 binds to JA–Ile (or COR) in the absence of JAZ. A role for COI1 and JAZ as coreceptors thus remains a formal possibility. Significantly, however, COI1 is predicted to adopt a structure that is similar to the auxin receptor TIR1 (Tan et al. 2007). Several amino acid residues that mediate the interaction of TIR1 with auxin, substrate, and inositol hexakisphosphate are conserved in COI1. The results of binding experiments performed with extracts from the tomato *jai1-3* mutant, which harbors a point mutation (L418F) in a region of COI1 that is homologous to the hormone-binding pocket of TIR1, suggests that this region of COI1 is important for interaction with the ligand and/or JAZ substrate. However, we cannot exclude the possibility that reduced binding of COR to *jai1-3* extracts results from an effect of the L418F mutation on the stability or abundance of COI1.

We found that the interaction of COI1 with two divergent members (JAZ1 and JAZ3) of the tomato JAZ family is promoted in a highly specific manner by JA–Ile and structurally related JA conjugates. It is noteworthy that JA–Val, whose synthesis is catalyzed by JAR1-like enzymes (Staswick et al. 2004, Wang et al. 2008), was as active as JA–Ile in stimulating COI1 binding to JAZ3. This finding indicates that JA–Val may function as an endogenous signal for COI1-dependent responses, particularly in tissues that contain high JA–Val levels. JA–Leu and JA–Ala also exhibited activity, suggesting that these derivatives are bioactive JAs as well. Although several studies have suggested that jasmonic acid, MeJA, and OPDA are active *per se* as signals in the JA pathway (Wang et al. 2008, Stintzi et al. 2001, Seo et al. 2001, Kramell et al. 1997, Koch et al. 1999) these

compounds showed no activity in the JAZ1 and JAZ3 pull-down assays. It is possible that these nonconjugated derivatives promote COI1 interaction with JAZ proteins whose ligand specificity is different from that described here for JAZ1 and JAZ3. The well-defined repertoire of JAZ proteins in model plants such as *Arabidopsis* indicates that it will be possible to systematically determine the signal specificity of all JAZs by using protein–protein interaction assays.

Several pathovars of *P. syringae* possess a cluster of genes that direct the synthesis of the phytotoxin COR (Bender et al. 1999). Here, we show that COR functions as an agonist of the JA receptor. Remarkably, COR is 1000-fold more active than JA–Ile in promoting the COI1–JAZ3 interaction *in vitro*. This is consistent with the fact that COR is generally a much more potent signal in physiological responses than is jasmonic acid or MeJA (Uppalapati et al. 2005, Koda et al. 1996). The virulence properties of COR can thus be attributed to its ability to efficiently promote SCF^{COI1}-mediated ubiquitination and destruction of JAZ proteins. This conclusion is supported by studies demonstrating that COI1-deficient mutants of *Arabidopsis* and tomato are much less susceptible to infection by COR-producing strains of *P. syringae* (Feys et al. 1994, Zhao et al. 2003). Targeting of eukaryotic hormone receptors by pathogen virulence factors would appear to provide an efficient mechanism to manipulate genome-wide transcriptional programs and other processes that effectively suppress host cell defenses. It is not yet clear whether the toxic properties of COR result solely from an overstimulation of the JA signaling pathway due to its high receptor-binding efficiency or whether COR has additional properties that alter normal cellular

processes.

Pull-down experiments with truncated forms of tomato JAZ3 showed that $JAZ3_{149–306}$ interacts with COI1 in a ligand-dependent manner, whereas a derivative consisting of the N-terminal 221 aa does not. This finding indicates that the sequence determinants for substrate recognition by SCF^{COI1} are located within the C-terminal 157 aa of JAZ3. Because the highly conserved Jas motif is located in this region, we suggest that the Jas motif mediates JAZ binding to COI1. This hypothesis is consistent with studies showing that deletion of the Jas motif stabilizes JAZ proteins against COI1-dependent degradation during JA signaling (Thines et al. 2007, Chini et al. 2007, Yan et al. 2007). Experiments conducted with *Arabidopsis* JAZ3, however, showed that the ZIM domain-containing N-terminal region, but not the C-terminal region containing the Jas motif, interacts with COI1 in the absence of exogenous JA (Chini et al. 2007). One possible explanation for these apparently disparate results is that COI1 interacts with the N and C termini of JAZ3 in a JA-independent and -dependent manner, respectively. Although we did not detect COI1–$JAZ3_{1–221}$ interaction in the absence of JA–Ile, our pull-down assay is likely less sensitive than the assay used by Chini et al. (2007), who used radiolabeled proteins for their binding studies.

In summary, our results build on recent studies (Thines et al. 2007, Chini et al. 2007, Yan et al. 2007, Chung et al. 2008) to define a unifying model for JA signaling in which direct recognition of JA–Ile by COI1 is coupled to ubiquitin-mediated degradation of JAZs and subsequent derepression of primary response

genes. The results extend the emerging paradigm (Tan et al. 2007, Dharmasiri et al. 2005, Kepinski et al. 2005) of F-box proteins as intracellular sensors of small molecules to trigger the destruction of regulatory proteins and suggest a common evolutionary origin of the JA and auxin response pathways.

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Chapter 4

Biochemical characterization of the jasmonate receptor

Abstract

Jasmonic acid (JA) and its derivatives, collectively known as jasmonates, are potent signals in growth regulation, reproductive development, and host immunity to insects and pathogens. Among the central components of the JA signaling cascade are the E_3 ubiquitin ligase SCF^{COI1} and JAZ proteins that repress transcription of JA-responsive genes. Recent studies provide evidence that the JA-amino acid conjugate jasmonoyl-isoleucine (JA-Ile) initiates signal transduction by promoting the formation of a stable complex between the F-box protein COI1 and JAZ. The bacterial toxin coronatine (COR) is a structural mimic of JA-Ile and a potent agonist of this hormone receptor system. Here we show that JA-Ile and COR also promote interaction between Arabidopsis COI1 and previously uncharacterized JAZ proteins. *In vitro* pull down experiments performed with purified AtCOI1 showed that COI1 alone is not a JA receptor. These findings show that ligand binding requires both COI1 and JAZ. Testing of isomers of JA-Ile in receptor binding assays have found that the orientation of the side-chains of JA-Ile affect its binding affinity. Pull down assays with JA-Ile derivatives identified structural features of JA-Ile and COR which promote a COI1-JAZ interaction and have provided insight into how the JA signal can be attenuated *in vivo*.

Introduction

Jasmonic acid is the classical member of a family of linolenic acid derived cyclized oxylipins, the jasmonates (JA). JAs regulate diverse aspects of plant defense, growth, and reproductive development (Browse 2005, Wasternack 2008). The vast majority of JA–mediated responses are dependent on a functional COI1 (Devoto et al. 2005). As the F-box component of the SCF^{COI} complex, COI1 establishes the specificity of the complex to bind to and target JAZ repressors for ubiquitin-mediated degradation (Thines et al. 2007, Chini et al. 2007). Degradation of JAZ proteins by COI1 activates the expression of JA-responsive genes. Mutations that impair the turnover of JAZ result in the constitutive repression of such genes (Thines et al. 2007, Chini et al. 2007). Receptor-binding studies show a COI1-JAZ complex is the site of binding for jasmonoyl-isoleucine (JA-Ile) and the phytotoxin coronatine (COR) (Katsir et al. 2008). Such studies have yet to resolve if COI1 can act as a receptor in the absence of JAZ but do indicate that COI1 is an essential component of the JA receptor.

It is unclear whether jasmonic acid is recognized at the site of JA perception. Rather, the emerging view is that JA-Ile is the primary ligand for the JA receptor. Both JA and JA-Ile accumulate rapidly in response to wounding (Chung et al. 2008, Suza et al. 2008). The rapid increase in both compounds correlates with the induction of JA-responsive genes (Chung et al. 2008). The characterization of *jar1* plants, partially insensitive to JA but highly responsive to JA-Ile, indicate that conjugation of JA to isoleucine is important for JA perception.

JA-Ile is primarily synthesized by the enzyme JAR1 that forms an isopeptide bond between JA and the free carboxyl of isoleucine (Staswick et al. 2004). Synthesis of JA-Ile is required for a robust defense response to some insects and pathogens (Staswick et al. 1998, Laurie-Berry et al. 2006, Kang et al. 2006). It is not yet clear that all JA responses are initiated by the perception of JA-Ile, as a plant devoid of JA-Ile has yet to be characterized.

JAZ proteins from both tomato and Arabidopsis interact with COI1 in a JA-Ile dependent manner. However, it is not known whether all JAZ proteins act in this way (Katsir et al. 2008, Thines et al. 2007, Melotto et al. 2008). The *JAZ* gene family in Arabidopsis contains twelve members, and in tomato seven *JAZ* genes have been identified (Thines et al 2007, Chini et al 2007, Katsir et al. 2008, Katsir and Howe unpublished). Sequence similarity among JAZ proteins is primarily limited to two signature sequence motifs. The TIF[F/Y]XG sequence is highly conserved in all JAZ proteins and is located within the so-called ZIM domain (Vanholme et al. 2007). Proteins that possess this sequence are part of a larger TIFY family which include the PEAPOD (PPD) proteins that regulate leaf development (White et al. 2006), as well as other members which contain zinc-finger DNA binding domains (Shikata et al. 2003). Arabidopsis JAZ1 and JAZ3 are located in the nucleus, however neither contains a known DNA binding domain (Chini et al. 2007, Thines et al. 2007). The C-terminal Jas motif $SLX_2FX_2KRX_2RX_5PY$ is the second distinguishing feature of the JAZ proteins (Chini et al. 2007, Thines et al. 2007). The C-terminal domain of JAZ, containing the Jas motif is necessary and sufficient for the formation of the COI1-JAZ

receptor complex (Katsir et al. 2008). Mutagenesis of conserved residues within the Jas motif indicates this sequence maybe the site of JAZ binding to COI1 (Melotto et al. 2008). It is also through its C-terminal domain that JAZ interacts with MYC2, a basic helix-loop-helix (bHLH) transcription factor that is a positive regulator of JA-responsive gene expression (Chini et al. 2008). Interestingly, point mutations in the Jas motif that abrogate JA-Ile stimulated COI1-JAZ binding does not effect the interaction of JAZ with MYC2 (Melotto et al. 2008).

The JA signalling pathway may have mechanisms to attenuate itself. The observation that *JAZ* genes are rapidly and transiently induced led to the hypothesis that JAZ proteins may act to repress their own synthesis (Thines et al. 2007). Resynthesis of JAZ proteins during the JA response would provide a negative feedback loop that could attenuate the responses. Both JA and JA-Ile levels begin to decrease after an initial burst in response to wounding possibly due to metabolism (Chung et al. 2008, Suza et al. 2008). Enzymatic modification of JA-Ile may be another route to attenuate the JA response. The fate of JA-Ile is poorly characterized. However, several derivatives of JA-Ile that may represent an inactivation pathway have been identified (Miersch et al. 2008). Hydroxylated (12-OH-JA-Ile) and di-carboxylated derivatives of JA-Ile have been detected in wounded plant tissue (Glauser et al. 2008). The phased accumulation of some of these compounds suggests that they are products of JA-Ile metabolism. In tomato, methyl-JA-Ile (Me-JA-Ile) has been detected in flowers (Hause et al 2000). It is not known if any of these compounds retain activity similar to that of JA-Ile or whether they represent distinct signalling molecules.

Here we have characterized several diverged JAZ proteins in Arabidopsis and have shown that they interact with COI1 in a JA-Ile and COR-dependent manner. To determine whether the JA receptor requires both COI1 and JAZ components acting together, rather than COI1 alone, we tested purified COI1 in a radio-ligand binding assay. Taking advantage of a small library of JA-Ile conjugates we are able to assess the active moieties of the core JA molecule. The loss of activity in some JA-Ile derivatives suggests modification of JA-Ile can attenuate the JA signal. These results also provide insight into the characteristics of COR that confer its strong activity compared to JA-Ile in promoting a COI1-JAZ interaction.

Materials and Methods

Biological Materials

Growth conditions for *Solanum lycopersicum* (tomato) and *Arabidopsis thaliana* were previously described (Li et al. 2001, Koo et al. 2006). A *35S-SlCOI1-Myc* transgenic line of tomato (cv Microtom) and a *35S-AtCOI1-Myc* transgenic line of Arabidopsis were used as sources of SlCOI1-Myc and AtCOI1-Myc, respectively, for pull-down experiments (Thines et al. 2007, Melotto et al. 2008).

Chemicals

Coronatine (C8115) and (±) jasmonic acid were purchased from Sigma. 12-OH-jasmonoyl-isoluecine (12-OH-JA-Ile), 12-oxo-phytodienoic acid isoleucine (OPDA-Ile), jasmonoyl-coranamic acid (JA-CMA) were synthesized and the resulting compounds were verified by GC-MS as described previously (Kramel et al 1998, Staswick et al. 2004). Isomers of (3R, 7R)-cucurbinoyl-isoluecine were prepared as reported previously (Kramel et al. 1999). The naturally occurring (-)-JA-Ile isomer was used in pull-down assays, whereas all other JA conjugates were used as a mixture of the (+)-JA- and (-)-JA-amino acid diastereomers. The synthesis of the four stereoisomers of JA-Ile was carried out by Dr. Yuichi Kobayashi (Department of Biomolecular Engineering, Tokyo Institute of Technology). A description of the synthesis and characteristics of [^{3}H]-COR was previously reported (Katsir et al. 2008).

Cloning and Expression of Fusion Proteins

The *AtJAZ1* cDNA was amplified by PCR using the primers JAZ1-Not1 (5'-GCGCGGCCGCCATGTCGAGTTCTATGGAATGTTCT-3') and JAZ1-Xho1 (5'-CCCTCGAGTATTTCAGCTGCTAAACCGAG-3'). The *AtJAZ3* cDNA was amplified using the primers AtJAZ3-Not1 (5'-GCGCGGCCGCCATGGAGAGAGATTTTCTCGGGTTG -3') and AtJAZ3-Xho1 (5'- CCCTCGAGGGTTGCAGAGCTGAGAGAAGAACT -3'). The *AtJAZ6* cDNA was amplified using the primers AtJAZ6-Not1 (5'-GCGCGGCCGCCACGGGACAAGCGC -3') and AtJAZ6-Xho1 (5' – CGGCTCGAGAAGCTTGAGTTCAAGGTTTTTGG -3'). The *AtJAZ7* cDNA was amplified using the primers AtJAZ7-Not1 (5'-GCGCGGCCGCCATCATCATCAAAAACTGCGACAAGCC -3') and AtJAZ7-Xho1 (5'- CGGCTCGAGTCGGTAACGGTGGTAAGG -3'). The *AtJAZ12* cDNA was amplified using the primers AtJAZ12-Not1 (5'-GCGCGGCCGCCGTGAAAGATGAGCCACG -3') and AtJAZ12-Sal1 (5'-TCGGTCGACAGCAGTTGGAAATTCCTCC -3') (restriction sites underlined). The PCR products were digested with NotI and XhoI (or Sal1 in the case of *AtJAZ12*) and cloned into the corresponding sites of pRMG-nMAL to produce MBP-JAZ-6XHis fusion products (Thines et al., 2007). Cloning of *SlJAZ1* and *SlJAZ3* into pRMG-nMAL was described previously (Katsir et al. 2008). Expression and purification of JAZ-fusion proteins was previously described (Thines et al. 2007, Katsir et al. 2008). GST-AtCOI1 and ASK1-6XHis (GST-AtCOI1) were co-expressed and purified to >90% homogeneity as described

previously for the expression and purification of TIR1 (Tan et al. 2007). Weak expression of AtJAZ7-His resulted in purification of this fusion protein to ~60% homogeneity (data not shown).

Pull-Down and [^{3}H]-COR Binding Assays

A *35S-SlCOI1-Myc* transgenic line of tomato and a *35S-AtCOI1-Myc* line of Arabidopsis were used as sources of SlCOI1-Myc and AtCOI1-Myc, respectively (Thines et al. 2007, Melotto et al. 2008). Unless otherwise indicated, the quantity of JAZ-His added to each reaction was 25 µg. For pull-down reactions with AtJAZ7-His, 50 µg of the purified protein was used. Protein concentrations were determined with a BCA Protein Assay kit (Pierce). [^{3}H]-COR binding assays contained 20 µg AtJAZ12-His, 2 µg GST-AtCOI1, and 500 nM [^{3}H]-COR in a final volume of 0.1 mL binding buffer (50 mM Tris pH 6.8, 10% glycerol, 100 mM NaCl, 0.1% Tween-20, and Complete Mini Protease Inhibitor tablet-EDTA free Roche) and were performed in triplicate. Binding specificity was determined by addition of 500 fold unlabeled COR.

Competitive binding reactions were supplemented with increasing concentrations of either unlabeled COR, one of the four JA-Ile isomers, or jasmonic acid. Reactions were incubated at 4°C for 30 min, after which 25 µl of glutathione resin (Pierce) was added. Following an additional 15-min incubation at 4°C, GST-AtCOI1-bound glutathione resin was washed three times on micro-centrifuge spin columns with 0.25 ml binding buffer at 4°C. GST-COI1 was eluted from the resin with 200 µl of a solution containing 10 mM reduced glutathione (Pierce). Radioactivity in the resulting eluent was measured by scintillation

counting after addition of 1 ml scintillation fluid (MicroBeta Trilux, Perkin Elmer).

Results

Arabidopsis JAZ proteins interact with COI1

Several Arabidopsis JAZ (AtJAZ) proteins, including AtJAZ1, AtJAZ3, and AtJAZ9 interact with Arabidopsis COI1 (AtCOI1) in a JA-Ile and COR dependent manner (Thines et al. 2007, Melotto et al. 2008). We used a similar pull-down assay to test the interaction between AtCOI1 and several additional AtJAZs that were expressed as MBP-6XHis fusion proteins (designated AtJAZ-His). To determine if JA-Ile is a ligand for other COI1-JAZ pairs, we tested three uncharacterized JAZ proteins (AtJAZ6, AtJAZ7, and AtJAZ12) that are diverged from two previously characterized JAZ proteins AtJAZ1 and AtJAZ3, which were included as positive controls.

The positive controls AtJAZ1-His and AtJAZ3-His, as well as AtJAZ12-His recovered AtCOI1-Myc above background levels in pull down assays supplemented with 1 μM JA-Ile (Thines et al. 2008, Melotto et al. 2008)

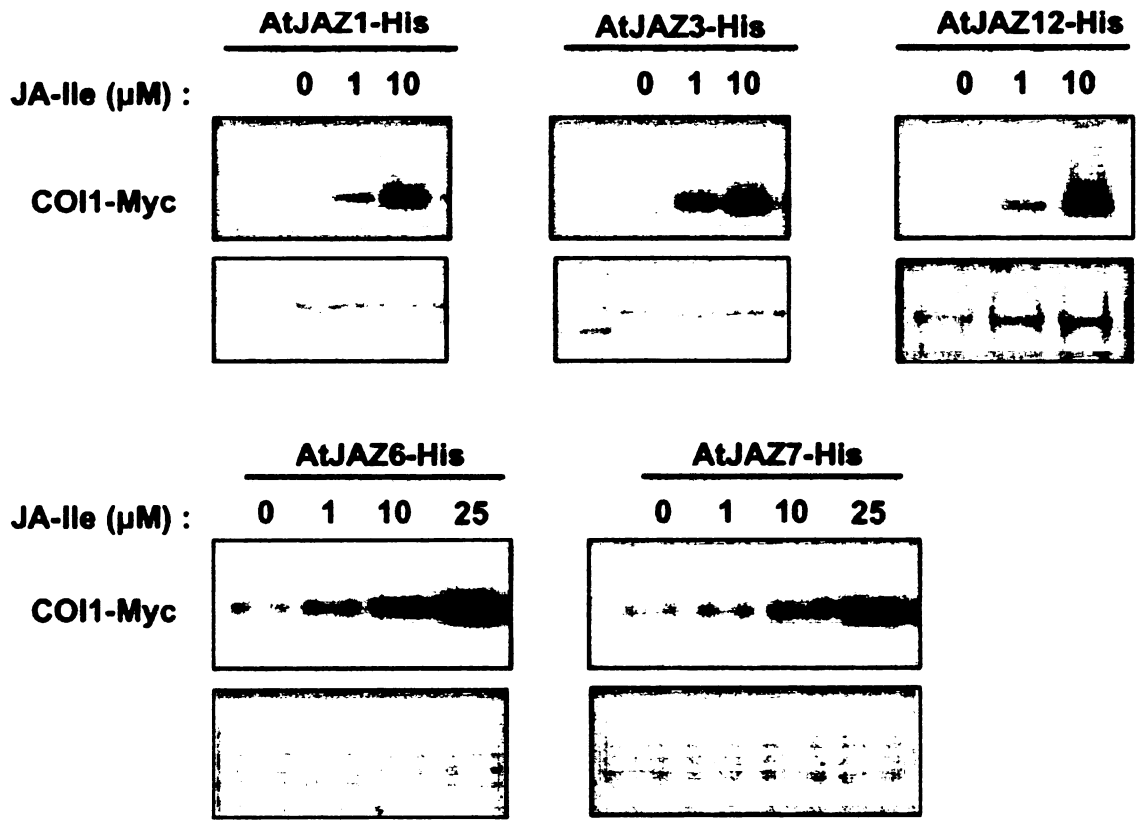

Figure 4.1. JA-Ile promotes JAZ interaction with COI1.

Pull-down assays were preformed by mixing extracts from *35S-AtCOI1–Myc* plants with recombinant AtJAZ1–His, AtJAZ3-His, AtJAZ6-His, AtJAZ7-His, or AtJAZ12-His Assays were supplemented various concentrations of JA-Ile and incubated for 30 min at 4°C. Protein bound to JAZ-His was analyzed by immunoblotting for the presence of AtCOI1–Myc. The Coomassie Blue-stained blot in each panel shows the recovery of JAZ-His by the Ni-affinity resin.

Higher concentrations of JA-Ile [10 μM] enhanced the recovery of AtJAZ1, AtJAZ3, and AtJAZ12, demonstrating the dose dependence of hormone induced binding. Binding of AtJAZ6-His to AtCOI1-Myc was only slightly enhanced by 1μM JA-Ile in comparison to the mock control (Figure 4.1). Recovery of AtCOI1-Myc by AtJAZ6-His was clearly enhance by higher concentrations of JA-Ile (10 and 25 μM). No binding above background levels was seen at 1 μM JA-Ile, only low amounts of AtCOI1-Myc were recovered in reactions containing 10 μM JA-Ile. Robust recovery of AtCOI1-Myc by AtJAZ7-His was observed in the presence of 25 μM JA-Ile. The weaker binding found in AtJAZ6-His and AtJAZ7-His pull downs may reflect a weak affinity for AtCOI1 for these proteins. It cannot be ruled out that the purity of AtJAZ7-His may account for its weak binding compared with other AtJAZ- proteins.

We next tested whether binding of AtJAZ1, 3, 6, 7, and 12 to AtCOI1 is stimulated by COR. All AtJAZ-His proteins assayed interacted with AtCOI1 in the presence of 1 μM COR (Figure 4.2). These results indicate that COR exerts its influence on JA signaling by promoting the interaction of COI1 with a majority of the JAZ proteins, rather than selectively targeting a small sub-set.

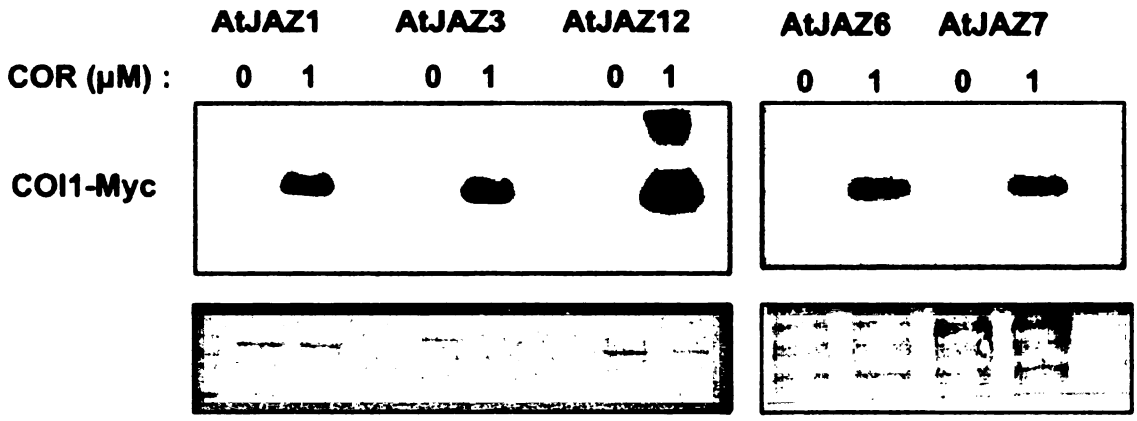

Figure 4.2. Coronatine promotes interaction with COI1 with several members of the Arabidopsis JAZ family.

Pull-down assays were preformed with recombinant JAZ1–His, JAZ3-His, JAZ6-His, JAZ7-His, or JAZ12-His and extracts from *35S-AtCOI1–Myc* plants. Assays were supplemented with various concentrations of COR and carried out as described in Figure 4.1. The Coomassie Blue-stained blot in each panel shows the recovery of JAZ-His by the Ni-affinity resin.

Ligand binding requires COI1 and JAZ

Previous studies showed that COI1 is an essential component of the receptor for COR and JA-Ile (Katsir et al. 2008). However, because these receptor-binding experiments depended on pull downs with JAZ protein we could not distinguish whether COI1 acts as a receptor on its own or whether JAZ was required for binding as well (Figure 4.3A). To address this question we collaborated with Dr. Ning Zheng's lab to obtain purified COI1 as a fusion to glutathione s-transferase (GST-COI1). The availability of purified COI1 allowed us to test the ligand binding characteristics of COI1 in the absence of JAZ. Pull down reactions containing both AtCOI1-GST and AtJAZ12-His specifically recovered [^{3}H]-COR (Figure 4.3C). Specific binding of COR to AtJAZ12 was not observed in the absence of COI1, consistent with previous work with SlJAZ3. When GST-COI1 was tested for its interaction with [^{3}H-]-COR, specific binding was not detected. The inability of [^{3}H-]-COR to bind specifically to GST-COI1 or JAZ suggests that COI1-JAZ may form a co-receptor complex. These results indicate that COI1 and JAZ are both necessary for ligand binding, and that the two proteins together are sufficient for binding.

COI1-JAZ is selective for JA-Ile isomers

Jasmonic acid possesses two chiral centers at the C-3 and C-7 positions, resulting in four possible isomers of jasmonic acid (Figure 4.4A). The biosynthetic pathway of jasmonic acid guides the natural product into a (+)-(3R, 7S) conformation, with the two side chains of the cyclopentenone ring in the *cis* arrangement.

Figure 4.3. COI1-JAZ is a co-receptor.

Two models for JA-Ile binding to a COI1-JAZ receptor complex are presented. A, COI1 first binds JA-Ile, which enhances COI1's ability to bind JAZ. B, Neither COI1 nor JAZ alone can bind to JA-Ile. The two proteins may have weak affinity for each other in the absence of ligand (Chini et al. 2007). JA-Ile enhances the affinity of COI1 for JAZ, resulting in a COI1-JA-Ile-JAZ complex. C, Pull-down reactions containing AtJAZ12-His, GST-AtCOI1, and [^{3}H]-COR were incubated in the presence (+) or absence (-) of 1000-fold unlabeled COR. The amount of radioactivity recovered by the GST-AtCOI1 complex is shown. Error bars denote the SD of triplicate assays.

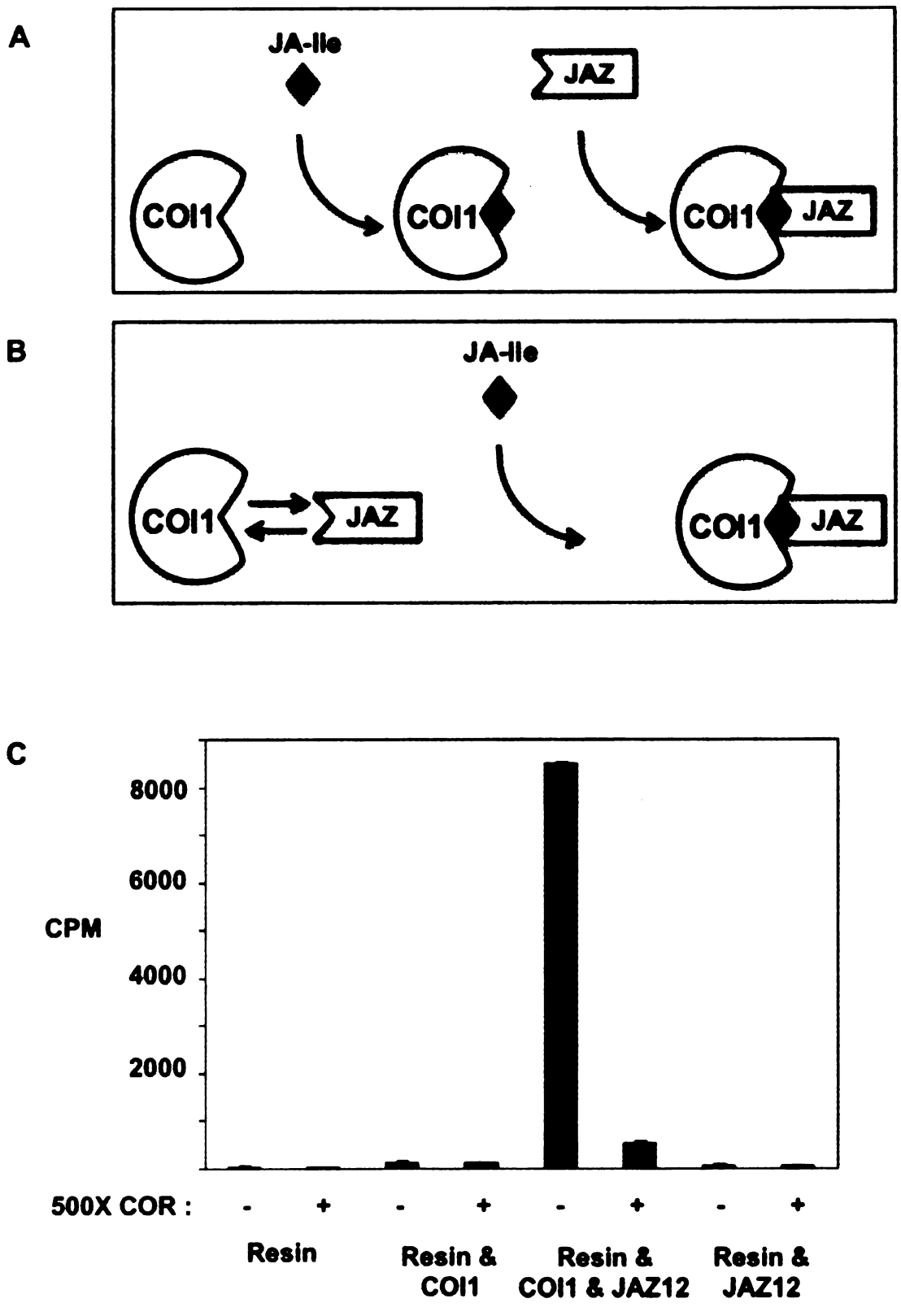

Figure 4.3. COI1-JAZ is a co-receptor.

The stereochemistry of jasmonic acid is established during cyclization by the enzyme allene oxide cyclase (Ziegler et al. 1999). The *cis* configuration of JA is less stable than JA with its side chains in *trans*. As a result the unstable *cis* configuration, (+)-(3R, 7S)-JA undergoes epimerization to produce the (-)-(3R, 7R)-JA isomer, which has its side chains in the *trans* orientation. Naturally synthesized jasmonic acid and JA-Ile is a mixture of isomers in the *cis* and *trans* conformations (Vick and Zimmerman 1984, Holbrook et al. 1997).

To understand how the isomeric configuration of JA-Ile affects binding to COI1-JAZ, we tested the ability of four isomers of JA-Ile to compete with [^{3}H]-COR in competition binding assays (Figure 4.4B). Unlabeled COR effectively competed for the GST-COI1-AtJAZ-His binding site. The EC50 (50% effective competition) for COR was 6 μM. The four JA-Ile isomers tested all competed with COR for the COI1-JAZ binding site, but to differing degrees. The natural product (3R, 7S)-JA-Ile and the (3S, 7S)-JA-Ile isomer exhibited EC50 values of 35 μM and 45 μM, respectively, that were six to seven fold higher than COR. The other two isomers, (3S, 7R)-JA-Ile and (3R, 7R)-JA-Ile, were ~60 to 130 (EC50 of 350 μM and 800 μM) fold less active than COR, respectively. The lack of competition with jasmonic acid is consistent with its inability to promote a COI1-JAZ complex.

Figure 4.4. Activity of JA-Ile isomers in competitive binding assays

A, Molecular structures of the four isomers of JA-Ile and COR. B, Pull-down reactions containing AtJAZ12 and GST-AtCOI1 were incubated with [^{3}H]-COR in the presence of increasing concentrations of unlabeled COR (●), (3R,7S; K1) JA-Ile (O), (3S, 7R; K2) JA-Ile (▲), (3R, 7R; K3) JA-Ile (△), (3S, 7S; K4) JA-Ile (■), and jasmonic acid (□). Radioactivity recovered with JAZ12-His is indicated (CPM). C, Pull down assays were performed with recombinant SlJAZ3-His and extracts from *35S-COI1-Myc* plants supplemented with COR or an isomer of JA-Ile at the concentration indicated. The Coomassie Blue-stained blot in the lower panel shows the recovery of protein by the Ni-affinity resin.

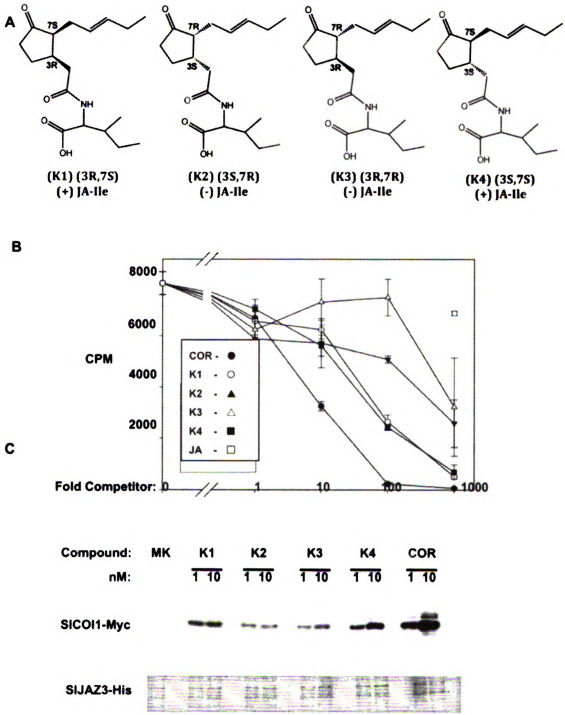

Figure 4.4. Activity of JA-Ile stereoisomers

Isomers of JA-Ile were also tested for their ability to promote a interaction between SlCOI1-Myc and SlJAZ3-His. The results showed pattern of activity similar to what was observed in [^{3}H]-COR competition assays is seen (Figure 4.4C). The most active of the isomers were (3R, 7S)- JA-Ile and (3S, 7S)-JA-Ile. These compounds stimulated the recovery of more SlCOI1 than the (3S, 7R)-JA-Ile and the (3R, 7R)-JA-Ile. The similarity of the isomer preference in Arabidopsis and tomato likely represent a conserved structural element that determines specificity.

Structure activity relationships of JA-Ile derivatives

We tested the ability of 12-OH-JA-Ile to promote SlCOI1 interaction with SlJAZ3 in a pull down assay (Figure 4.5B). This modification of the pentenyl moiety of JA-Ile had no effect on the amount of COI1-Myc recovered by SlJAZ3-His. However, the interaction between SlCOI1 and SlJAZ1-His was greatly reduced in reactions supplemented with 12-OH-JA-Ile compared to JA-Ile. This indicates that hydroxylation of the pentenyl side chain could potentially promote some COI1-JAZ interactions while blocking others. These results also reinforce previous observation that SlJAZ3 is less discriminating than SlJAZ1 with its ligand preference (Katsir et al. 2008).

Figure 4.5. Binding activity of various JA-Ile derivatives

A. Molecular structures of 12-OH-JA-Ile, cucurbinoyl-isoleucine, OPDA, and OPDA-Ile. B, COI1-Myc pull-down assays were preformed with SlJAZ1–His or SlJAZ3-His. Assays were supplemented with JA-Ile (JI), 12-OH-JA-Ile (OH-J), (6S)-curubinoyl-(S)-Ile (C1), (6R)-cucurbinoyl-(S)-Ile (C2), (6S)-cucurbinoyl-(R)-Ile (C3), or (6R)-cucurbinoyl-(R)-Ile (C4). C, COI1-Myc pull-down assays were preformed with SlJAZ1–His or SlJAZ3-His COI1-Myc and supplemented with JA-Ile (J-Ile), OPDA, or OPDA-Ile (O-Ile) at the indicated concentration. The Coomassie Blue-stained blot in the lower panel shows the recovery of protein by the Ni-affinity resin.

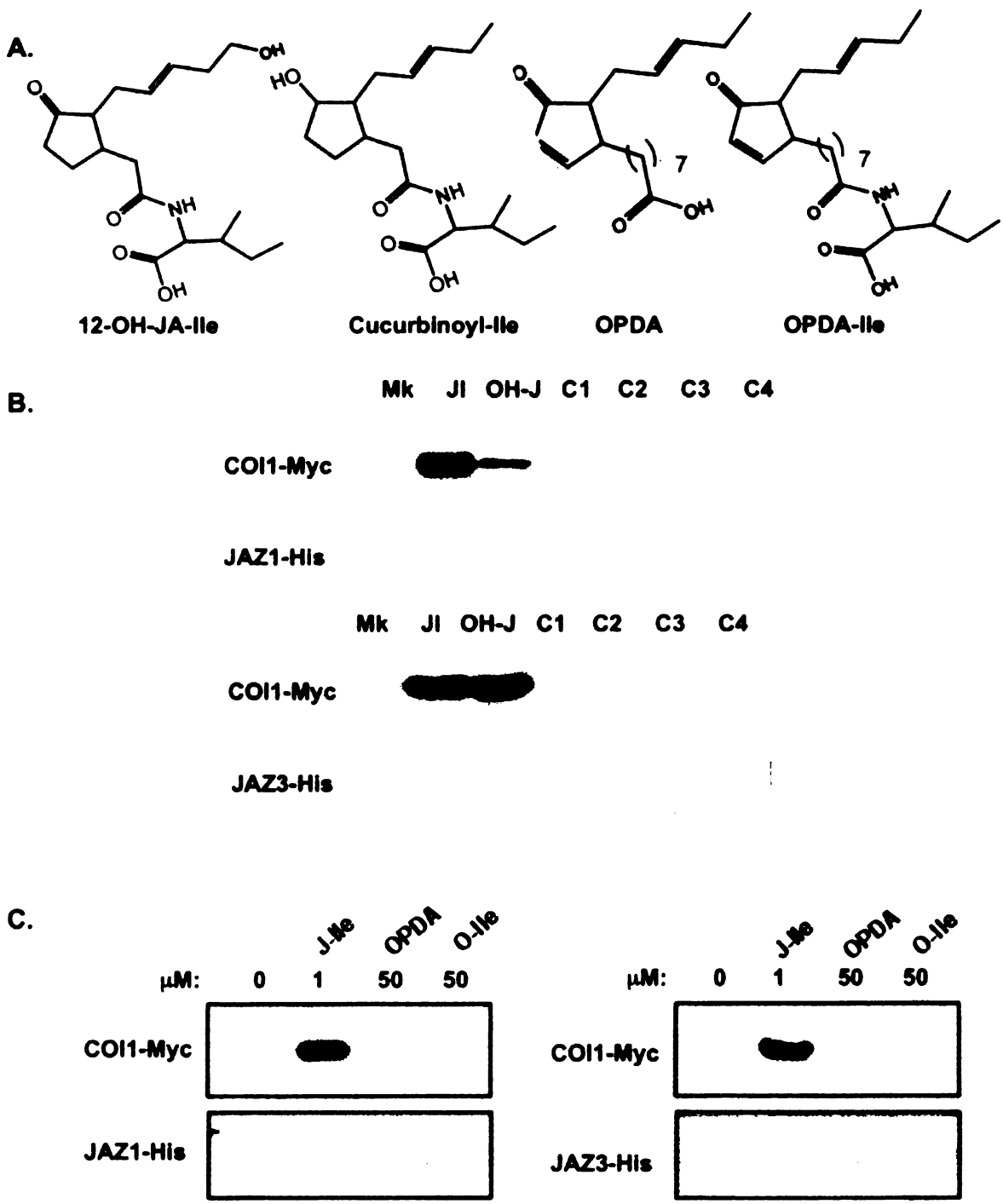

Figure 4.5. Binding activity of various JA-Ile derivatives.

The cyclopentanone ring is another distinguishing feature of JA-Ile on COR (Figure 4.5A). To test the importance of this substituent in the COI1-JAZ interaction, we compared the activity of cucurbinoyl-Ile (CA-Ile) to that of JA-Ile in SlCOI1 pull down assays. CA-Ile is a JA-Ile derivative in which the ketone of the cyclopentanone ring is replaced by an alcohol (Figure 4.5A). Four isomers of CA-Ile representing epimers of the cyclopentanol conjugated to either (R) or (S) Ile were tested (Figure 4.5B). No recovery of COI1-Myc above background was detected in pull downs conducted with SlJAZ1 or SlJAZ3 in the presence of 1μM CA-Ile, whereas the same concentration of JA-Ile stimulated a robust recovery of COI1-Myc. These results demonstrate that the ketone group on the cyclopentanone ring is essential for hormone-induced COI1-JAZ interaction and that Ile-conjugated derivatives of cucurbic acid are not likely to signal through COI1.

The JA biosynthetic precursor 12-oxo-phytodienoic acid (OPDA) has been implicated as a signal for COI1-dependent responses (Stintzi et al. 2001, Ribot et al. 2008). However, OPDA does not promote a COI1 interaction with any tomato or Arabidopsis JAZ proteins tested to date (Thines et al. 2007, Katsir et al. 2008, Melotto et al. 2008). One possibility is that OPDA is conjugated to Ile to enhance its activity, though an OPDA-Ile conjugate has never been detected in plants. This compound also provides a tool to test the importance of Ile proximity to the cyclopentenone ring (Figure 4.5A). To test this idea, the activity of OPDA and OPDA-Ile was compared to JA-Ile in a COI1-Myc pull down assay (Figure 5.4C).

The results showed that OPDA-Ile was unable to promote COI1 interaction with either SlJAZ1-His or SlJAZ3-His at concentrations 50 times higher than JA-Ile.

We previously reported that COR is 100 to 1000-fold more active than JA-Ile in promoting COI1-JAZ interactions in tomato (Katsir et al. 2008). COR is a conjugate of coronafacic acid (CFA) and coronamic acid (CMA) (Figure 4.6A). It was also shown that both the CFA and the CMA moieties of COR lack the ability to promote a COI1-JAZ interaction (Melotto et al. 2008). To address the basis of COR's enhanced activity we tested the activity of a jasmonate-COR chimera consisting of jasmonic acid conjugated to CMA (Figure 4.6A, B). The results show that both SlJAZ1-His and SlJAZ3-His recovered slightly less SlCOI1 in the presence of JA-CMA compared to JA-Ile. This result indicates that the CMA moiety of COR is not sufficient to account for the enhanced ability of COR (relative to JA-Ile) in promoting COI1-JAZ binding.

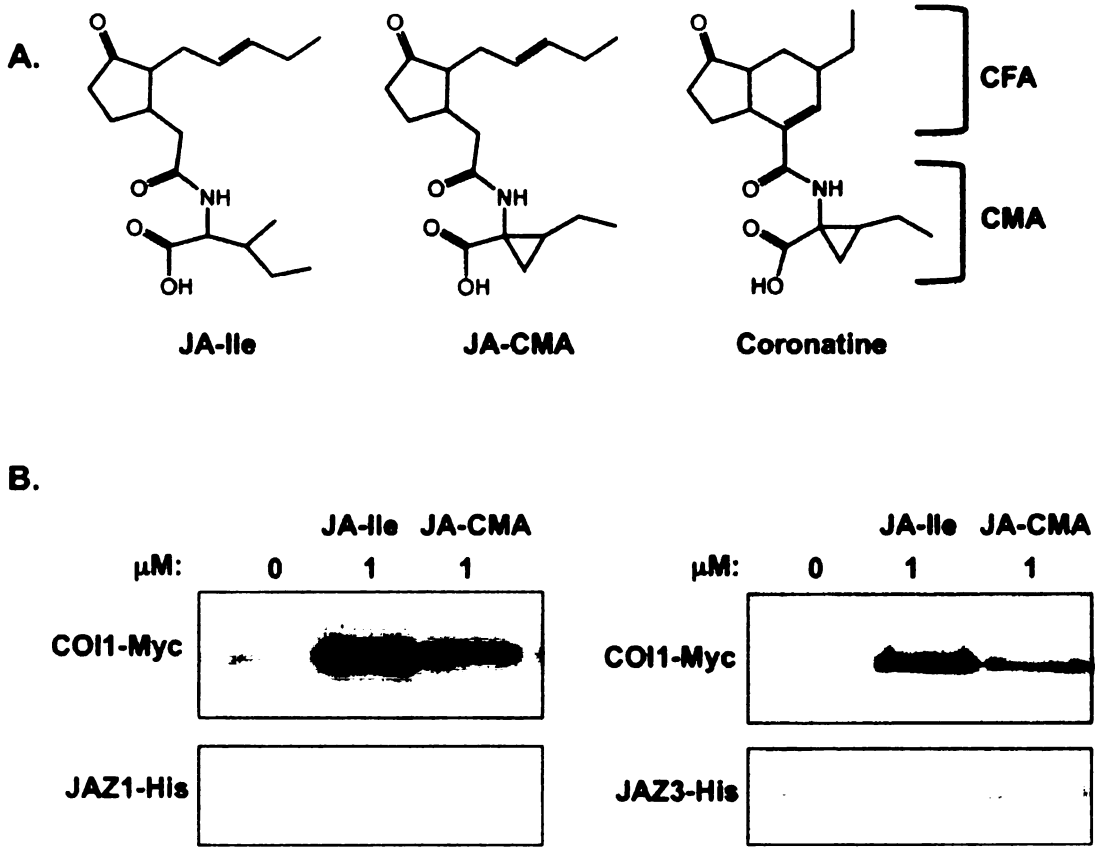

Figure 4.6. Comparison of JA-Ile and JA-CMA

A, The molecular structures of JA-Ile, JA-CMA, and coronatine. B, Pull-down assays were preformed with SlJAZ1–His and SlJAZ3-His and extracts from *35S-SlCOI1–Myc* plants. Assays were supplemented with 1 μM JA-Ile or JA-CMA. The Coomassie Blue-stained blot in the lower panel shows the recovery of protein by the Ni-affinity resin.

Methylation of JA-Ile reduces its activity in promoting a COI1-JAZ interaction.

Me-JA-Ile was previously shown to accumulate in tomato flowers, suggesting that this compound may play a role in JA signaling (Haues et al. 2000). We compared the activity of Me-JA-Ile to that of JA-Ile in COI1-Myc pull down assays with SlJAZ1-His and SlJAZ3-His (Figure 4.7). At a concentration of 1 μM, MeJA-Ile promoted the recovery of COI1-Myc by both SlJAZ1 and SlJAZ3. The activity of JA-Ile was at least 10-fold higher than that of Me-JA-Ile. These results suggest that the methylation of JA-Ile may serve as a way to attenuate the intensity of the JA-Ile signal.

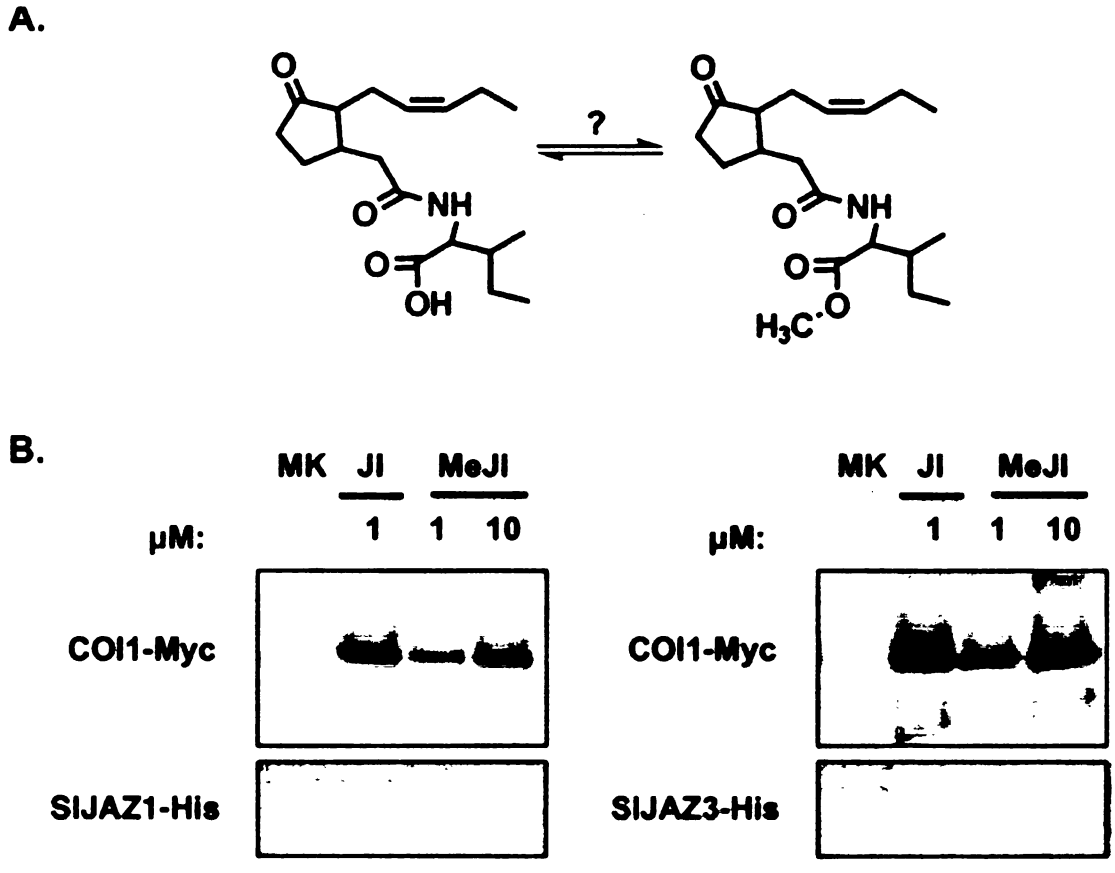

Figure 4.7. Methylation of JA-Ile reduces its activity.

A, The structures of JA-Ile and Me-JA-Ile are shown in a reversible reaction carried out by unknown enzymes. B, Pull-down assays were preformed with SlJAZ1–His or SlJAZ3-His and extracts from *35S-SlCOI1–Myc* plants. Assays were supplemented with JA-Ile (JI) and Me-JA-Ile (MeJI) at the indicated concentrations.

Discussion

Our results add to a growing consensus that interaction of JAZ proteins with COI1 is promoted by JA-Ile (Thines et al. 2007, Melotto et al. 2008, Katsir et al. 2008). Among the 12 JAZ proteins in Arabidopsis, JAZ1, JAZ3, JAZ6, JAZ7, JAZ9, and JAZ12 have been shown to interact with COI1 in the presence of JA-Ile and the JA-Ile mimic, COR (Thines et al. 2007, Melotto et al. 2008). In tomato, two diverged JAZ proteins also interact with COI1 in a JA-Ile dependent manner (Katsir et al. 2008). The presence of the highly conserved Jas motif, which is required for COI1-JAZ binding in all JAZ proteins, supports the idea that other JAZs have a similar preference for JA-Ile (Katsir et al. 2008, Melotto et al. 2008). Until all JAZ proteins are characterized, however, the possibility that some JAZs do not interact with COI1 cannot be ruled out. The regulatory functions of JAZ proteins likely involves JAZ binding partners such as MYC2 (Chini et al. 2007).

Previously we showed that SlJAZ may be more permissive than SlJAZ1 in its ligand-mediated interaction with COI1. Here, we found that the affinity for binding COI1 in the presence of JA-Ile was reduced for AtJAZ6 and AtJAZ7 compared with other JAZ proteins. This observation suggests that some JAZ interact with COI1 in response to low concentrations of JA-Ile, whereas other JAZs interact with COI1 when JA-Ile concentrations are very high. The existence of JAZ proteins with different binding affinities could provide plants with a mechanism to tune their responses to the relative level of JA-Ile signal.

Our results also provide new insight into the mechanism of JA-Ile perception by COI1-JAZ. Previously we reported that COI1 or a COI1-JAZ complex is a receptor for JA-Ile and COR (Katsir et al. 2008). These studies could not discriminate between models in which ligand binding is mediated by COI1 alone or whether binding involves a COI1-JAZ complex. We found that COI1 cannot bind COR in the absence of JAZ and that both COI1 and JAZ together are required for a functional receptor complex. Because TIR1 binding to auxin in the absence of Aux/IAA has not been demonstrated, this conclusion is not fundamentally different from what has been reported for TIR1-Aux/IAA binding to auxin. One possible model for COI1-JAZ binding to JA-Ile (Figure 4.2B) is that CO1 and JAZ have very weak hormone independent affinity for each other and are in constant equilibrium (Chini et al. 2007). Increases in JA-Ile concentrations would shift this equilibrium toward COI1-JAZ complex formation when JA-Ile interacts with the two proteins. It cannot be ruled out that JA-Ile binds to COI1 with an affinity that is below our ability to detect it. Biophysical approaches will be required to further investigate how small molecules promote this novel mode of receptor-ligand binding.

Enzymatic modification of JA-Ile may be involved in the attenuation of JA signaling. Epimerization of newly synthesized jasmonic acid may also represent a mechanism for tuning down the JA response. Jasmonic acid is synthesized in the unstable *cis* conformation that naturally epimerizes to the trans conformation by a keto-enol tautomerization (Holbrook et al. 1997). As a result of this epimerization it is likely that JA-Ile is composed of both *cis* and *trans* isomers.

The isomeric composition of JA-Ile and jasmonic acid *in planta* is unknown. Competitive binding assays with the four isomers of JA-Ile showed that the side chain orientation of JA-Ile affects the formation of the COI1-JAZ complex. We found that the natural *cis* isomer (+) (3R, 7S) of JA-Ile was ~20-fold more effective at competing with COR than the corresponding *trans* isomer (-) (3R, 7R) JA-Ile. We also showed a similar preference for specific JA-Ile isomers to promote COI1-JAZ interactions in tomato. Thus, epimerization of JA-Ile has a profound effect on its activity, and suggests epimerization may act as a natural timer for deactivation of the JA signal.

Although the enzymes that hydroxylate JA and JA-Ile have not been identified, the presence of such hydroxylated compounds in several plant species has been demonstrated (Yoshihara et al. 1989, Swiatek et al. 2004, Glauser et al. 2008). The physiological significance of JA hydroxylation has been primarily attributed to the tuber inducing properties of tuberonic acid (12-OH-JA). The emerging view of hydroxylated jasmonic acid and JA-Ile is that they represent intermediates in a pathway for inactivation of the JA signal (Miersch et al. 2008). Hydroxylation is also route to further enzymatic modifications, resulting in a diversity of JA conjugates linked through the C12 position (Swiatek et al. 2008). We found that 12-OH-JA-Ile promotes the interaction between tomato JAZ proteins and COI1. Interestingly, 12-OH-JA-Ile was more effective in promoting a COI1 interaction with SlJAZ3 than with SlJAZ1. This result leads us to suggest that hydroxylation reduces the activity of JA-Ile. The contribution of the cyclopentanone ring of JA-Ile was also investigated with isomers of cucurbinoyl-

isoleucine. The results of these experiments demonstrate an absolute requirement for this functional group, which is also present at an equivalent position of the COR structure.

We found that the CMA conjugate of JA was not more active than JA-Ile. This result implies that the CFA portion of COR is largely responsible for the high potency of the toxin. Indanoyl-isoleucine conjugates have an activity similar to COR, also suggesting that the CFA moiety confers the enhanced activity (Fleigman et al. 1995). JA-Ile isomers with the pentenyl side chain in the (7S) orientation were more active than the (7R) JA-Ile (Figure 4.4). This may be due to the orientation of this side chain relative to the ketone group of the cyclopentanone ring. The COR ring structure is locked in an equivalent conformation, which may explain why the activity of COR more closely resembles the activity of JA-Ile molecules with their side chain in the (7R) orientation.

We found that methylation of JA-Ile at its free carboxylic acid decreases COI1-JAZ binding. Because Me-JA-Ile is produced in plants, methylation of JA-Ile may be a way to attenuate the signal (Hause et al. 2000). Alternatively, methylation of JA-Ile may facilitate its intra or intercellular transport as a less active signal until arrival in appropriate target tissue where demethylation of Me-JA-Ile promotes JAZ degradation. The detection of Me-JA-Ile in flowers suggests that this molecule may play a role in reproductive development (Hause et al. 2000).

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Chapter 5

Conclusions and future perspectives

Major conclusions

The discovery that JAZ proteins are substrate of SCF^{COI1} is a watershed mark in JA research. COI1-JAZ binding assays provided distinct evidence that the hormone acts to promote COI1-JAZ binding and that this activity requires the conjugation of jasmonic acid to Ile (Thines et al. 2008). That jasmonic acid is not an active ligand for any of the COI1-JAZ pairs tested to date is remarkable because jasmonic acid was long considered to be the active form of the hormone (Thines et al. 2007, Katsir et al. 2008, Melotto et al. 2008). The windfall of discoveries surrounding the identification of JAZ substrates included a description of the mechanism by which COR activated JA signaling to promote *P. syringae* virulence. The strength of COR binding also facilitated the characterization of the COI1-JAZ complex as a receptor for COR and JA-Ile (Katsir et al. 2008). Further biochemical characterization revealed that COI1 and JAZ are both required for ligand binding, highlighting the complexity of this new class of receptor.

Unanswered questions and future directions

Many unanswered questions concerning the mechanism of JA perception remain to be addressed. The finding that both COI1 and JAZ are required for ligand binding has important implications for how F-box proteins, including TIR1, perceive small molecules. One possibility is that at low levels of JA, COI1 and JAZ interact weakly but that their association is unstable (Figure 4.1B). This idea is supported by the low level recovery of COI1 in JAZ-His pull down assays performed in the absence of exogenous ligand, and the ligand-independent COI1-JAZ interaction reported by Chini et al. (Chini et al. 2008). A weak,

hormone-independent COI1-JAZ interaction may serve to maintain JAZ in close proximity to SCF^{COI1}. As JA-Ile levels increase, the interaction between COI1 and JAZ would be stabilized via the Jas motif, presumably in a manner similar to auxin-mediated TIR1-Aux/IAA binding (Thines et al. 2007, Tan et al. 2007). Crystal structures of the COI1-JA-JAZ complex, together with ligand binding kinetic and affinity data acquired through the use of more sensitive biophysical techniques, such as measuring protein-protein interaction by surface plasmon resonance (Biacore), will increase our understanding of this novel type of hormone-receptor binding.

Binding of JA to a COI1-JAZ complex presumably frees the TFs (i.e. MYC2) that are repressed by JAZ proteins in the absence of hormone. It is currently unclear whether JAZ proteins are general suppressors of transcription, recruited to the promoter of JA-responsive genes by a cognate TF (i.e. MYC2), or if JAZ proteins repress transcription by blocking their cognate TF from interacting with DNA. Testing the influence of JAZ proteins in reporter gene assays and determining the influence of JAZ on MYC2-DNA binding could help discriminate how TF activity is repressed by JAZ. JAZ recruitment to the promoters of JA responsive genes would also imply that COI1 is recruited to those sites as well.

The finding that JAZs interact with both COI1 and MYC2 raises the possibility that COI1 and MYC2 compete for JAZ binding and that JA influences the binding equilibrium between these players. It was recently shown that two amino acids in the Jas motif that are essential for COI1-JAZ interaction are not required for JAZ-MYC2 binding (Melotto et al. 2008). Identifying specific regions

of JAZ that are important for MYC2 binding may reveal the basis for COI1-induced dissociation of JAZ-MYC2. Binding studies to determine the affinity of different JAZ-MYC2 pairs may reveal specificity of some JAZ proteins for MYC2 over others. It is also possible that a strong JAZ-MYC2 interaction impedes JA-mediated binding of COI1 to JAZ. DNA binding and protein-protein interaction studies will be necessary to reveal the dynamics of the multiple components of JA signaling.

One of the major questions in JA biology is how SCF^{COI1}-mediated degradation of JAZ stimulates specific responses, such as reproductive development and defense. Critical to understanding this question is the identification of additional-JAZ interacting proteins. The repressive function of JAZ indicates that these proteins act to suppress the activity of TFs involved in activating the JA response (Thines et al. 2007, Chini et al. 2007). In addition to MYC2, the involvement of other TFs is predicted by the fact that JA signaling is not completely abolished in plants lacking MYC2 (Lorenzo et al. 2004). The identification of other JAZ-TF partners may reveal a role for specific TFs in distinct responses. Cell specific expression of particular JAZ-TF pairs may also be an important strategy to organize specific JA responses in a specific tissue type. This would make sense in reproductive tissue where developmental signals relayed by JA, would need to be kept distinct from defense responses.

The ability to discriminate between severe attack and more mild damage may be advantageous to plants in natural environments. One reason for this is the growth penalty incurred when a defense response is mounted (Howe and

Jander 2008). JA signaling may be tuned to generate an appropriate response. One hypothesis is that the extent of JAZ degradation is graded in response to increasing levels of JA. The binding properties of different COI1-JAZ pairs that control different sets of genes may be determined by precise intracellular concentrations of JA, rather than a mechanism in which a threshold level of JA acts as an on/off switch. Thus, in Arabidopsis where there are 12 different JAZ proteins and a single COI1, there may be 12 distinct COI1-JAZ receptors for the hormone. A complete biochemical evaluation of different COI1-JAZ pairs could determine whether these receptors have distinct JA binding affinities. Physiological detection of dose-dependent JAZ degradation could be accomplished by monitoring the degradation of JAZ proteins with JAZ-specific antibodies. Fluorescence resonance energy transfer (FRET) may provide another approach for *in vivo* monitoring of the dose specific interaction between COI1 and specific JAZ proteins.

The JA signaling pathway must be turned off upon cessation of the environmental stress that generated the signal. For example, the transcript level of JA early responsive genes that are rapidly induced by wounding begins to decrease three hours after the wound stimulus (Chung et al. 2008). It has been hypothesized that JAZ proteins synthesized in response to JA re-accumulate and act to repress their own gene expression, as a means of shutting down JA responses (Thines et al. 2008, Chini et al. 2008). JA levels peak close to one hour after wounding and then begin to decrease but remain above background levels for at least 8 hours (Chung et al. 2008, Suza et al. 2008). In order to shut

off JA responses in wounded tissue where JA-Ile levels remain high, JAZ proteins may be rendered resistant to SCF^{COI1} mediated degradation (Chung et al. 2008, Suza et al. 2008). One explanation for wound-induced synthesis of JAZ repressors that are unresponsive to JA-Ile levels is the expression of alternatively spliced JAZ variants (i.e. AtJAZ10) that lack the Jas domain and cannot interact with COI1 (Yan et al. 2007). Another intriguing possibility is that phosphorylation or other post-translational modifications may modify JAZ proteins to block their interaction with COI1 in wounded plants. A JAZ protein has been identified as a target of phosphorylation and there is evidence that phospho-transfer has a role in JA signaling (Katou et al. 2005, Rojo et al. 1998).

Enzymatic and non-enzymatic factors contribute to the generation of an active JA signal and also to signal attenuation. In this thesis I have defined an active JA signal as a compound that promotes the interaction between COI1 and a JAZ protein. The finding that JA-Ile promotes COI1-JAZ interaction strongly supports the idea that the conjugation of JA to Ile is a requirement for JA activity (Staswick et al. 2004). However, until a mutant plant completely devoid of JA-Ile is generated, it cannot be determined whether JA-Ile is required for all JA-mediated responses. In the course of this study I have identified a variety of jasmonoyl-amino acid conjugates and JA-Ile derivatives that promote a COI1-JAZ interaction. Plants lacking the ability to synthesize JA-Ile could be used to monitor physiological responses to specific JAs, as well as for monitoring the accumulation of alternative JAs in response to wounding. In this thesis, the activity of several modifications to JA-Ile, including 12-hydroxylation, methylation,

and isomerization from *cis* to *trans* JA-Ile were described. It can be speculated that certain modifications (e.g. methylation) that change the physical properties of JA-Ile may enhance the hormones mobility within or between cells. It is also possible that in some cases the site of JA production and perception are distinct, and that enzymes at the site of signal generation make JA-Ile inert for transit to the site of perception. The importance of ligand modification to the attenuation of the JA signal is an active and exciting area of research.

As with every important discovery, characterization of the JAZ proteins has provoked many more questions than its discovery has answered. Uncovering the mode of JA perception will have important implications for the way in which we consider the mechanism of regulation of all JA mediated responses. In a broader context understanding the basis of JA perception is critical to understanding how the hundreds of other F-box proteins in plants sense other hormones and environmental stimuli.

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