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A critical role of two positively charged amino acids in the Jas motif of Arabidopsis JAZ proteins in mediating coronatine- and jasmonoyl isoleucine-dependent interactions with the COI1 F-box protein

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Summary

Coronatine is an important virulence factor produced by several pathovars of the bacterial pathogen *Pseudomonas syringae*. The structure of coronatine is similar to that of a class of plant hormones called jasmonates (JAs). An important step in JA signaling is the SCF^{COI1} E3 ubiquitin ligase-dependent degradation of JAZ repressor proteins. We have recently shown that jasmonoyl isoleucine (JA-Ile) promotes physical interaction between Arabidopsis JAZ1 and COI1 (the F-box component of SCF^{COI1}) proteins, and that the JA-Ile-dependent COI1–JAZ1 interaction could be reconstituted in yeast cells (i.e. in the absence of other plant proteins). Here we show that coronatine, but not its two biosynthetic precursors, also promotes interaction between Arabidopsis COI1 and multiple JAZ proteins. The C-terminal Jas motif, but not the N-terminal (NT) domain or central ZIM domain of JAZ proteins, is critical for JA-Ile/coronatine-dependent interaction with COI1. Two positively charged amino acid residues in the Jas domain were identified as essential for coronatine-dependent COI1–JAZ interactions. Mutations of these two residues did not affect the ability of JAZ1 and JAZ9 to interact with the transcription factor AtMYC2. Importantly, transgenic Arabidopsis plants expressing JAZ1 carrying these two mutations exhibited JA-insensitive phenotypes, including male sterility and enhanced resistance to *P. syringae* infection. These results not only suggest that coronatine and JA-Ile target the physical interaction between COI1 and the Jas domain of JAZ repressors, but also illustrate the critical role of positively charged amino acids in the Jas domain in mediating the JA-Ile/coronatine-dependent JAZ interaction with COI1.

Keywords: coronatine, jasmonate, COI1, JAZ, plant immunity, *Pseudomonas syringae*.

Introduction

Jasmonates regulate a wide range of biological processes in plants, from sexual reproduction to herbivore defense and pathogen responses (Browse, 2005; Browse and Howe, 2008; Howe and Jander, 2008). A critical component of JA signaling is COI1, which is the F-box protein subunit of SCF^{COI1}, a member of the Skip/Cullin/F-box (SCF) family of E3 ubiquitin ligases (Li *et al.*, 2002; Xie *et al.*, 1998). E3 ubiquitin ligases are involved in the ubiquitination of specific protein substrates, targeting them to the 26S proteasome for degradation. The cognate substrates of SCF^{COI1} have been identified recently, and are members of the JAZ (jasmonate ZIM-domain) protein family (Chini *et al.*, 2007; Thines *et al.*, 2007; Yan *et al.*, 2007). Several JAZ proteins, including JAZ1, JAZ3 and JAZ6, have been

shown to be degraded in Arabidopsis in response to JA treatment, and this degradation is dependent on SCF^{COI1} and the 26S proteasome (Chini *et al.*, 2007; Thines *et al.*, 2007).

All JAZ proteins contain two highly conserved sequence motifs: the TIF[F/Y]XG signature that defines the so-called ZIM domain (Vanholme *et al.*, 2007) in the central portion of the protein, and the C-terminal Jas motif with the consensus sequence $SLX_2FX_2KRX_2RX_5PY$ (Yan *et al.*, 2007). In addition, Thines *et al.* (2007) noticed a weakly conserved region at the N-terminus (referred to as domain or NT). Moreover, mutant or transgenic plants expressing JAZ1, JAZ3 or JAZ10 lacking the Jas domain exhibit constitutive repression of JA signaling and are insensitive to JA (Chini *et al.*, 2007; Thines *et al.*, 2007; Yan *et al.*, 2007). Taken together, these results suggest that the Jas domain controls the stability of JAZ proteins in response to a JA signal in an SCF^{COI1}-dependent manner, whereas NT and ZIM domains are involved in repressing JA signaling. However, the specific mechanisms by which NT, ZIM and Jas domains control JAZ stability and/or repressor function are not yet understood.

Coronatine is produced by several pathovars of the bacterial pathogen *Pseudomonas syringae*. This toxin plays multiple roles in disease development – suppressing plant defenses locally and systemically, and inducing disease-associated tissue necrosis and chlorosis (Bender *et al.*, 1999; Block *et al.*, 2005; Brooks *et al.*, 2005; Cui *et al.*, 2005; Melotto *et al.*, 2006, 2008; Mittal and Davis, 1995; Underwood *et al.*, 2007). The overall structure of coronatine, consisting of coronafacic acid (CFA) and coronamic acid (CMA; Bender *et al.*, 1999), shows structural similarity to that of jasmonates (JAs), most notably jasmonyl isoleucine (JA-Ile; Staswick, 2008). Indeed, coronatine induces a large number of JA-responsive genes and other JA-related responses in plants (Bender *et al.*, 1999; Feys *et al.*, 1994; Lauchli and Boland, 2003; Thilmony *et al.*, 2006; Uppalapati *et al.*, 2005 Zhao *et al.*, 2003). In addition, the virulence function of coronatine requires COI1 (Feys *et al.*, 1994; Kloek *et al.*, 2001; Zhao *et al.*, 2003). Thus, it has been hypothesized that coronatine mimics jasmonates to exert its virulence function (Feys *et al.*, 1994). However, the specific host targets of coronatine have not yet been identified.

Chini *et al.* (2007) recently showed that purified recombinant JAZ3 interacts with COI1 produced in a wheatgerm *in vitro* transcription/translation extract in the absence of exogenous jasmonates. In these experiments, the N-terminal/central regions (containing the NT and ZIM domains) of JAZ3 were sufficient for interaction with COI1, whereas the C-terminus (containing the Jas domain) interacted with AtMYC2, a transcription factor known to be involved in JA signaling. However, Thines *et al.* (2007) showed that JAZ1 interaction with COI1 required JA-Ile. Other commonly used jasmonates, such as jasmonic acid (JA), methyl jasmonate (MeJA) or 12-oxo phytodienoic acid (OPDA), were not effective in promoting physical interaction between COI1 and JAZ1. The JA-Ile-dependent interaction between COI1 and JAZ1 was observed in protein pull-down assays using total plant protein extracts, as well as in yeast cells expressing COI1 and JAZ1 (i.e. in the absence of any other plant proteins), strongly suggesting that the COI1–JAZ1 complex is a site for JA-Ile perception. However, it is currently unclear whether the JA-Ile dependence represents a unique situation for the COI1–JAZ1 interaction or is a general mechanism applicable to COI1 interaction with other JAZ proteins. Similarly, it is not known which domain in JAZ proteins mediates JA-Ile-dependent interaction with COI1.

In this study, we show that (i) coronatine mimics JA-Ile in its ability to promote the physical interaction between COI1 and JAZ proteins, (ii) the requirement for JA-Ile or coronatine is a general mechanism for COI1 interaction with multiple JAZ proteins, (iii) two positively charged amino acid residues in the Jas domain are essential for coronatine-dependent COI1–JAZ interactions, (iv) Mutations of these two residues do not affect the ability of JAZ1 and JAZ9 to interact with the transcription factor AtMYC2, and (v) transgenic Arabidopsis plants expressing JAZ1 carrying these two mutations exhibit JA-insensitive phenotypes, including enhanced resistance to *P. syringae* infection.

Results

Coronatine promotes interaction between COI1 and JAZ1

To test the hypothesis that coronatine functionally mimics JA-Ile and facilitates formation of the COI1–JAZ1 complex independent of other plant proteins, we examined COI1–JAZ1 interaction in yeast cells. As shown in Figure 1(a), coronatine effectively promoted COI1–JAZ1 interaction. Neither CFA nor CMA, the two non-functional precursors of coronatine biosynthesis (Brooks *et al.*, 2005), had this activity, highlighting the importance of an intact coronatine structure for both biological activity and promotion of the COI1–JAZ1 interaction in yeast cells (Figure 1a).

COI1 interaction with JAZ1 could result in degradation of JAZ1 in yeast cells if the Arabidopsis COI1 protein were able to form a functional E3 ubiquitin ligase complex through interaction with heterologous yeast SCF subunits. To examine this possibility, we analyzed the expression of COI1 and/or JAZ1 proteins by Western blot analysis. As shown in Figure 1(b), co-expression of COI1 with JAZ1 did not lead to degradation of JAZ1 with or without coronatine, suggesting that COI1 does not form a functional E3 ubiquitin ligase in yeast cells, or, if such a functional ligase is formed, the JAZ1 protein is not degraded by the yeast 26S proteasome.

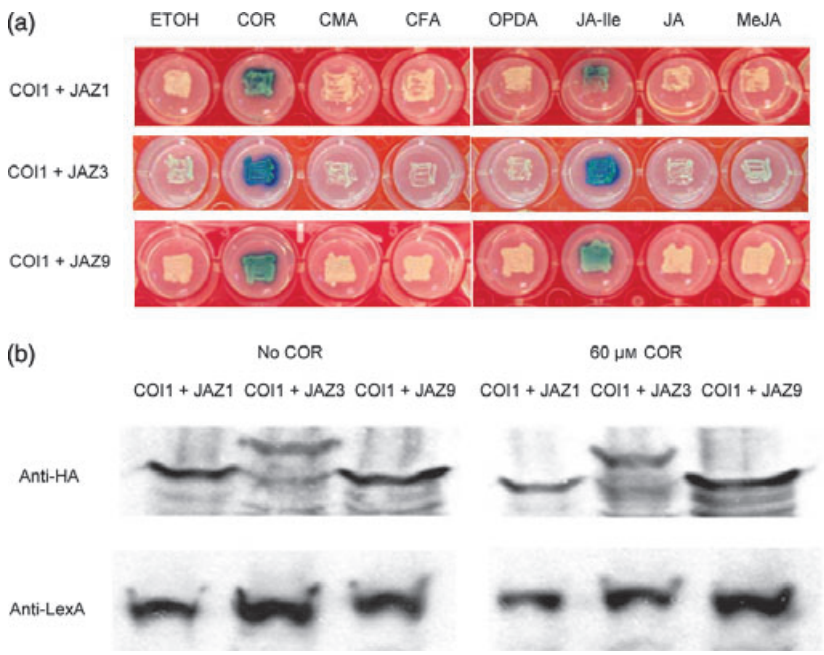

Figure 1. JA-Ile/coronatine-dependent interaction between COI1 and JAZ proteins in yeast.
(a) Yeast strains carrying pGILDA-COI1 and pB42AD-JAZ were streaked on Y2H agar medium (placed in wells of a microtiter plate). Blue colony color indicates a positive interaction; white colony color indicates no interaction. The chemicals coronatine (COR), JA, MeJA, JA-Ile, OPDA, CMA or CFA were added to the medium at a concentration of 60 μM. Ethanol is the solvent for the chemicals used. Images were obtained at day 4.
(b) Western blotting analysis of the yeast strains used in (a) to detect protein expression. The COI1 and JAZ proteins expressed from pGILDA-COI1 and pB42AD-JAZ were detected as fusion proteins using anti-LexA and anti-HA antibodies, respectively. Each lane was loaded with total protein extract from one strain.

The JA-Ile/coronatine-dependent interaction is not unique to the COI1–JAZ1 interaction

Yeast two-hybrid (Y2H) screens of an Arabidopsis cDNA library (Holt *et al.*, 2002) for coronatine-dependent COI1 interactors also independently yielded JAZ1 as well as several other JAZ proteins. Specifically, JAZ9 (formerly COI1-interacting protein 1; Melotto and He, 2007) clones were recovered in the Y2H screen in which the medium was supplemented with 1.5 μM coronatine, and JAZ1, JAZ2 and JAZ3 clones were isolated in the presence of 50 μM coronatine (data not shown). Furthermore, either coronatine or JA-Ile could promote these interactions, whereas JA, MeJA, OPDA, CFA or CMA did not (see Figure 1a for COI1–JAZ3 and COI1–JAZ9 interactions). Thus, the requirement for JA-Ile or coronatine is not unique to the COI1–JAZ1 interaction, but extends to several JAZ family proteins. Western blot analysis showed that co-expression of COI1 with JAZ3 or JAZ9 did not lead to degradation of JAZ3 or JAZ9 in yeast cells in the presence of coronatine (Figure 1b), again similar to what was observed for the COI1–JAZ1 interaction.

We next determined the concentrations of coronatine required for detection of various COI1–JAZ interactions. A concentration of coronatine of as low as 1.5 μM was sufficient to detect the COI1–JAZ9 interaction, whereas 30 μM coronatine was required to detect the COI1–JAZ1 interaction (Figure 2). However, expression of JAZ1 appeared to be toxic to yeast cells, based on the slow yeast growth, which could have negatively affected detection of this interaction, especially at lower coronatine concentrations. We also compared the relative concentrations of coronatine and JA-Ile required for detection of the COI1–JAZ9 interaction in yeast. Coronatine appears to be more effective than JA-Ile in these assays (Figure 2), suggesting that either coronatine is more potent than JA-Ile in promoting COI1–JAZ interactions, or yeast cells take up and/or metabolize coronatine and JA-Ile to varying extents.

In a previous study, recombinant tomato *Sl*JAZ1 protein [fused to maltose-binding protein (MBP) and the six-histidine (6xHis) epitope tag] was used successfully to pull down the tomato *Sl*COI1 protein (fused to the Myc epitope tag) from total tomato leaf extract in the presence of JA-Ile (Thines *et al.*, 2007). In this study, we performed similar protein pull-down assays using Arabidopsis COI1 and JAZ proteins. As shown in Figure 2(b), the COI1 protein (fused to the Myc epitope tag) in Arabidopsis leaf extract could be pulled down using the recombinant JAZ9 protein (fused to MBP and the 6xHis epitope tag). Moreover, the recombinant Arabidopsis JAZ1 protein (fused to MBP and the 6xHis tag) could be used to pull down tomato *Sl*COI1 (fused to the Myc tag), suggesting cross-species similarities in COI1–JAZ interactions (Figure 2b).

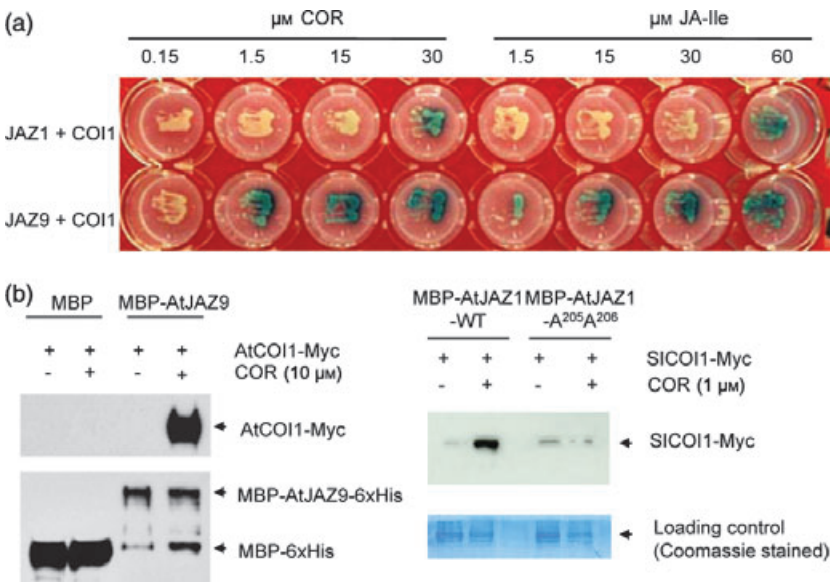

Figure 2. Y2H and protein pull down assays of COI1-JAZ interactions.
(a) The dose–response relationships for COI1 interaction with JAZ1 and JAZ9 in the presence of coronatine (COR) or JA-Ile (see Figure 1a for experimental set-up).
(b) Protein pull-down assays. Left, purified recombinant Arabidopsis JAZ1 protein (fused to MBP and 6xHis) was used to pull down COI1–Myc fusion protein from total Arabidopsis leaf extract in a coronatine (COR)-dependent manner. As a control, purified recombinant MBP–6xHis protein did not pull down COI1–Myc. COI1–Myc and MBP–JAZ–6xHis were detected by Western blotting using Myc epitope and MBP antibodies, respectively. Right, purified recombinant Arabidopsis JAZ1 protein (fused to MBP and 6xHis) was used to pull down *Sl*COI1–Myc fusion protein from total tomato leaf extract in the presence of COR. In contrast, purified recombinant MBP–JAZ1-A205A206–6xHis protein did not pull down *Sl*COI1–Myc. *Sl*COI1–Myc was detected by Western blotting using the Myc epitope antibody.

The C-terminal Jas domain, but not the NT or ZIM domain, is necessary and sufficient for interaction between COI1 and multiple JAZ proteins

Chini *et al.* (2007) have recently shown that the Arabidopsis JAZ3 protein interacted with COI1 without exogenous jasmonates, and furthermore that COI1 interacts with the N-terminal and central portions (including the NT and ZIM domains) of the JAZ3 protein. However, our sequence analysis of 32 COI1-interacting JAZ9 clones identified in the Y2H screening showed that C-terminal polypeptides excluding the ZIM domain were sufficient for coronatine-dependent interaction with COI1 (Supplementary Figure S1). To determine whether the C-terminus-dependent interaction was unique to JAZ9 or a general feature of COI1–JAZ interactions, we constructed several precise deletion derivatives of JAZ1, JAZ3 and JAZ9, and analyzed their abilities to interact with COI1. Deletion of the NT and/or ZIM domain in JAZ1 or JAZ9 did not affect the interaction with COI1 (Figure 3b). Expression of COI1 and these truncated JAZ proteins was detected in yeast (Figure 3c,d). Furthermore, we found that the C-terminus was sufficient for coronatine-dependent interaction between COI1 and JAZ1, JAZ3 and JAZ9 (Figure 3b,d). We conclude that it is the C-terminal Jas domain, not the NT or ZIM domain, that mediates JA-Ile- and coronatine-dependent JAZ interaction with COI1.

Identification of specific amino acid residues in the Jas domain that are important for COI1–JAZ1 interaction

Because the Jas motif is the only conserved sequence in the C-terminal region of JAZ1, JAZ3 and JAZ9, we conducted further mutagenesis experiments to identify specific amino acid residues in this motif that are important for COI1–JAZ interaction. We have previously shown that approximately 25% of T_1 transgenic Arabidopsis plants overexpressing a truncated JAZ1 lacking the entire Jas domain (JAZ1Δ3A) exhibited JA insensitivity, phenocopying *coi1* mutants (i.e. appearance of a male-sterile phenotype and increased resistance to *Pst* DC3000 infection; Thines *et al.*, 2007). To find out whether smaller changes in the Jas domain could also produce the same phenotype, we generated ten variants of the *JAZ1* cDNA, encoding proteins with smaller deletions or alanine substitutions in the Jas domain. When expressed in wild-type plants under the control of the 35S promoter, several of these mutations resulted in T_1 populations with significant proportions (4–16%) of male-sterile primary transgenic plants. In particular, a modified cDNA encoding a JAZ1 mutant protein in which R205 and R206 are both replaced by alanine residues (JAZ1-A205A206) produced 15 male-sterile plants within a tested T_1 population of 93 plants (Table 1). As expected, there were no sterile plants among those transformed with the vector alone. Male-sterile JAZ1-A205A206 plants produced seed when pollinated with

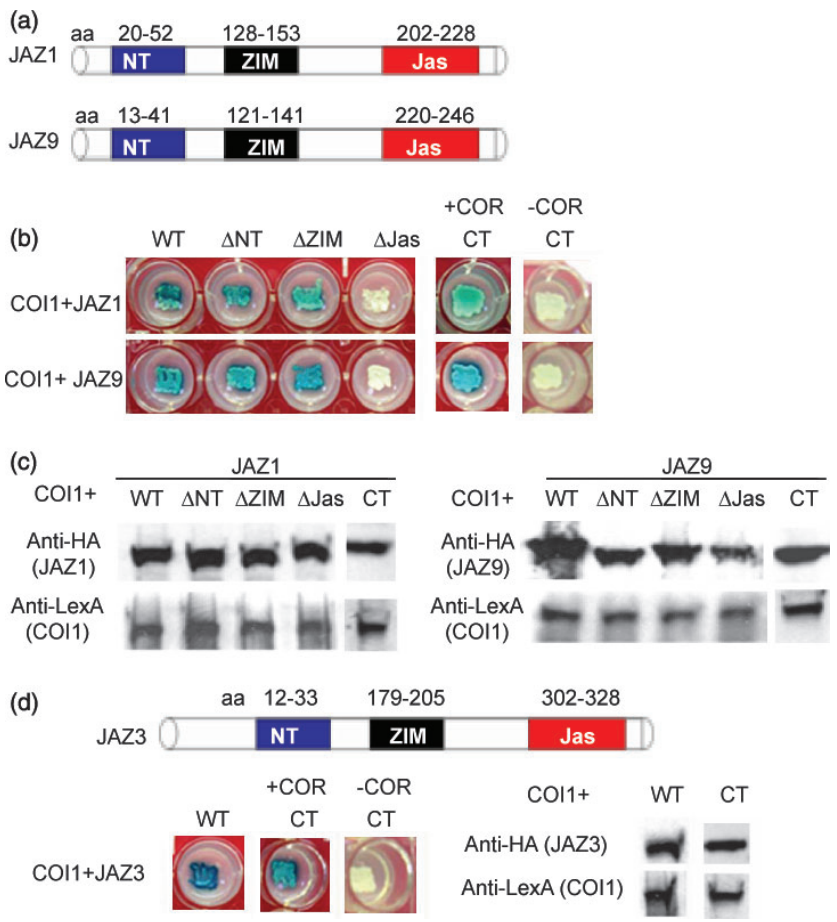

Figure 3. Requirement of the Jas domain for coronatine-dependent interaction between COI1 and JAZ1, JAZ3 and JAZ9.
(a) Domains (NT, ZIM, Jas) of the JAZ1 and JAZ9 proteins. Numbers indicate the amino acid positions in respective JAZ proteins.
(b) Y2H assay of JAZ1 and JAZ9 interaction with COI1. Blue colony color indicates a positive interaction; white colony color indicates no interaction.
(c) Western blotting analysis of the yeast strains expressing COI1 in combination with various JAZ1 and JAZ9 constructs. Each lane was loaded with total protein extract from one strain, and probed with anti-HA to detect the JAZ1 and JAZ9 proteins or anti-LexA to detect COI1 protein.
(d) JAZ3 Y2H and Western blot results for JAZ3 [see (a)–(c) for experimental set-up].
Western blot results for WT, ΔNT, ΔZIM and ΔJas of JAZ1 and JAZ9 were from the same experiment, whereas Western blot results for the Jas domain-containing C-terminus (CT) of JAZ1, JAZ3 and JAZ9 were from a separate experiment.

wild-type pollen. Among the T_2 progeny, individuals inheriting the transgene were male-sterile and those lacking the transgene were fertile. Moreover, the transgenic T_2 progeny were significantly more resistant to *Pst* DC3000 infection, compared with wild-type Col-0 plants, as reflected by reduced bacterial multiplication (Figure 4). Together, these results suggest that specific mutations in the Jas domain can confer JA-insensitive phenotypes similar to those of the *coi1* mutant plants.

To investigate the possibility that the residues R205 and R206 are required for Jas domain-mediated interaction with COI1, we created A205, A206 and A205A206 double mutations in pB42AD-JAZ1 and conducted Y2H assays to detect interaction with COI1 in yeast. All three mutations disrupted the COI1–JAZ1 interaction (Figure 5b). Furthermore, unlike the wild-type MBP–JAZ1–6xHis fusion protein, the MBP–JAZ1-A205A206–6xHis fusion protein failed to interact with tomato *Sl*COI1 in protein pull-down assays (Figure 2b).

We next examined whether corresponding mutations in another JAZ protein have the same or different effects. The residues R223 and K224 in JAZ9 (corresponding to R205 and R206 in JAZ1, respectively) were substituted with alanine residues. Like the A205A206 mutations, the A223A224 double mutation abolished the COI1–JAZ9 interaction, but single substitution mutations did not (Figure 5). Immunoblotting analysis showed that mutant JAZ1 and JAZ9 proteins were expressed (Figure 5b). Taken together, these results suggest that R205 and R206 in JAZ1 and R223 and K224 residues in JAZ9 play a critical role in mediating JA-Ile/coronatine-dependent COI1–JAZ interactions, and that alanine substitution mutations at these residues are sufficient to confer a dominant-negative effect on JAZ1 function in JA signaling (Table 1 and Figure 4).

Table 1 Male-sterile phenotype of T_1 transgenic plants expressing JAZ1 with deletion or amino acid mutations

Transgene	No. sterile plants	Total number of plants	Percentage sterile plants	Y2H interaction	Jas domain sequences (relevant amino acid residues underlined)
JAZ1				+	TELPIARRASLHRFLEKRKDRVTSKAPYQCDP
JAZ1Δ3	15	56	26.8	–	TEL————————————————CDP
JAZ1-A205A206	15	93	16.1	–	TELPIAAAASLHRFLEKRKDRVTSKAPYQLCDP
Empty vector	0	80	0	N/A	

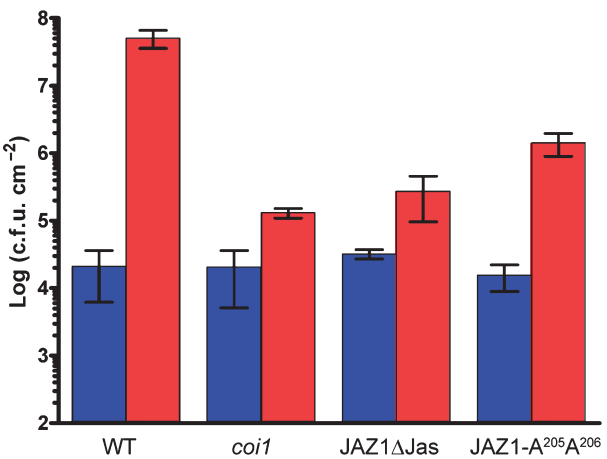

Figure 4. Susceptibility of Arabidopsis plants to *Pst* DC3000 infection. A suspension (1×10^6 colony-forming units per ml) of *Pst* DC3000 bacteria was syringe-infiltrated into Col-0 (WT), the *coi1* mutant and transgenic plants transformed with JAZ-ΔJas (JAZ1 with the entire Jas domain deleted) or JAZ1-A205A206 (JAZ1 in which R205 and R206 were replaced with alanine residues; see Figure 5a). Bacterial populations in leaves were shown as colony-forming units (c.f.u) cm^{-2} of leaf area at day 0 (blue) and day 3 (red) post-inoculation, with means and standard deviation shown ($n = 3$). The bacterial growth experiment was repeated twice.

Coronatine-independent interaction between JAZ and AtMYC2 proteins

The transcription factor AtMYC2 plays an important role in JA signaling, and was previously shown to interact with JAZ3 *in vitro* and in Y2H assays, establishing an important connection between SCF^{COI1}-dependent degradation of JAZ3 and gene expression in JA signaling (Chini *et al.*, 2007). The C-terminus containing the Jas domain was identified as the AtMYC2-interacting domain (Chini *et al.*, 2007). However, it is not known whether the AtMYC2–JAZ interaction is unique to JAZ3 or is a general phenomenon that applies to other JAZ proteins. To address this question, we conducted Y2H assays with JAZ1 and JAZ9 proteins. As shown in Figure 4(c), AtMYC2 interacted with both JAZ1 and JAZ9. Unlike the COI1–JAZ1/3/9 interactions, interaction between AtMYC2 and JAZ1 and JAZ9 proteins could be observed even in the absence of coronatine in the medium (Figure 5c); addition of coronatine or JA-Ile did not affect these interactions (data not shown). These results confirm that JAZ interaction with AtMYC is JA-Ile/coronatine-independent.

Because AtMYC2 interacts with the C-terminus (including the Jas domain) of JAZ3 (Chini *et al.*, 2007), we investigated whether R205/R223 (JAZ1/JAZ9) and R206/K224 (JAZ1/JAZ9) in the Jas domain are also involved in AtMYC2–JAZ interactions. As shown in Figure 5(c), these mutations did not affect AtMYC2 interaction with JAZ1 or JAZ9, demonstrating the specificity of the effects of these mutations on COI1–JAZ interactions.

Discussion

In this study, we addressed several questions arising from the recent identification of JAZ repressor proteins as the substrates of the SCF^{COI1} E3 ubiquitin ligase in JA signaling. First, we show that the bacterial toxin coronatine could effectively promote physical interaction between Arabidopsis COI1 and JAZ proteins. Among biologically active jasmonates commonly used in the study of JA signaling (e.g. JA, MeJA, OPDA and JA-Ile), only JA-Ile could promote COI1–JAZ interactions (Figure 1a; Thines *et al.*, 2007). Interestingly, the chemical structure of coronatine is similar to that of JA-Ile, and consists of CFA (coronafacic acid) and CMA (an ethylcyclopropyl amino acid derived from isoleucine; Bender *et al.*, 1999; Staswick, 2008). We found that neither CFA nor CMA promoted detectable COI1–JAZ interactions in yeast cells (Figure 1a). Together, these results highlight the importance of the Ile (or modified Ile) moiety in JA signaling and coronatine action, and suggest that coronatine is a potent microbial mimic of JA-Ile, targeting a key step of JA signaling, the formation of COI–JAZ complexes, as part of its virulence mechanism.

Another major conclusion from this study is that it is the C-terminus, not the N-terminus or central region, that is both necessary and sufficient for the interaction of several Arabidopsis JAZ proteins with Arabidopsis COI1 in a JA-Ile/coronatine-dependent manner (Figure 3). The C-terminal 157 amino acids of tomato *Sl*JAZ3 (including the Jas motif) are also necessary and sufficient for hormone-dependent binding of tomato *Sl*COI1 (Katsir *et al.*, 2008). However, it was not clear whether the Jas motif itself or another sequence in the C-terminus is important for *Sl*COI1–*Sl*JAZ3 interaction. In this study, we conducted further analyses with precise deletion of three individual intramolecular motifs of Arabidopsis JAZ1 and JAZ9, and show that neither the N-terminal motif nor the central ZIM motif is required,

(a)

JAZ1	JAZ1 domain 3 sequence	JAZ9	JAZ9 domain 3 sequence
WT	PIARRASLHRFLEKRKDRVTSKAPYQL	WT	PQARKASLARFLEKRKERLMSAMPYKK
R205A	PIAARASLHRFLEKRKDRVTSKAPYQL	R223A	PQAAKASLARFLEKRKERLMSAMPYKK
R206A	PIARAASLHRFLEKRKDRVTSKAPYQL	K224A	PQARAASLARFLEKRKERLMSAMPYKK
RR205/206AA	PIAAAASLHRFLEKRKDRVTSKAPYQL	RK223/224AA	PQAAAASLARFLEKRKERLMSAMPYKK

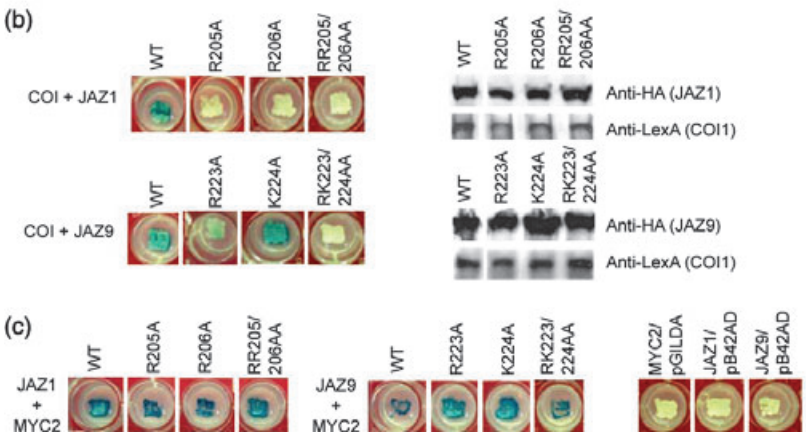

Figure 5. The role of Jas domain amino acid residues (R205R206 in JAZ1 and R223K224 in JAZ9) in JAZ interactions with COI1 and AtMYC2.
(a) The amino acid sequences of wild-type (WT) and mutated Jas domain of JAZ1 and JAZ9. Mutated residues are in red and underlined.
(b) Y2H (left panels) and Western blot (right panels) assays of COI1 interaction with WT or mutant JAZ1 or JAZ9 protein.
(c) Y2H assay of AtMYC2 interaction with WT or mutant JAZ1 protein or with empty Y2H vector (pGILDA or pB42AD).
To detect COI1–JAZ interaction (b), we streaked yeast strains on Y2H agar medium containing 60 μM coronatine (COR; see Figure 1 for experimental set-up). Interaction between JAZ and MYC2 does not require JA-Ile or coronatine; therefore, for (c), yeast strains were streaked on plain Y2H medium agar. Blue colony color indicates a positive interaction; white colony indicates no interaction.

but the Jas motif is (Figure 3). Furthermore, we performed site-directed mutagenesis in the Jas motif and have identified two basic amino acid residues (R205R206 in JAZ1 and R223K224 in JAZ9) in the Jas domain that are required for interaction of JAZ1 and JAZ9 with COI1 (Figure 5). By transgenic expression, we showed that these two positively charged residues are critical for JA signaling, as transgenic expression of JAZ1 carrying alanine substitutions of R205R206 conferred JA-insensitive phenotypes, including male sterility (Table 1) and enhanced resistance to *Pst* DC3000 infection (Figure 4). These results emphasize the importance of the Jas domain in coordinating molecular interactions of four key regulators of JA signaling: COI1 (a subunit of the SCF^{COI1} E3 ubiquitin ligase), JAZs (repressors of JA signaling), AtMYC2 (a transcription factor) and JA-Ile/coronatine (ligands; Figure 6).

As in the case of JA-Ile, promotion of COI1–JAZ interactions by coronatine was found to occur in yeast cells without requiring any other plant proteins. The ability of coronatine and JA-Ile to reconstitute COI1–JAZ interactions in heterologous yeast cells strongly implicates the CO11–JAZ complexes as receptors for these ligands. In support of this notion, ligand-binding experiments showed a critical role for *Sl*COI1 as part of a receptor for coronatine and JA-Ile (Katsir *et al.*, 2008). It is therefore likely that the initial step of JA signaling may mirror that of auxin signaling, in which auxin serves as 'molecular glue' for the physical interaction between the TIR1 F-box protein and the AUX/IAA repressor protein (Tan *et al.*, 2007). However, the requirement for positively charged residues in the Jas domain (e.g. R205R206 in JAZ1 and R223K224 in JAZ9) for interaction with COI1 suggests that the molecular nature of the COI1–JAZ interaction in JA signaling may be different from that of the TIR1–IAA interaction in auxin signaling. For the latter interaction, hydrophobic residues seem to play a predominant role (Tan *et al.*, 2007). For example, the IAA7 degron peptide, with the central hydrophobic consensus motif GWPPV for TIR1 binding, has a predominantly hydrophobic sequence and binds to the auxin-bound TIR1 pocket through extensive hydrophobic interactions (Tan *et al.*, 2007). In contrast, the requirement for positively charged residues for COI1–JAZ interactions suggests a critical role of electrostatic interactions.

Based on other findings (Chini *et al.*, 2007; Katsir *et al.*, 2008; Thines *et al.*, 2007) and the results obtained in this study, we propose a revised model for JA signaling (Figure 6). In the absence of JA-Ile or coronatine, the C-terminus of JAZ proteins binds to AtMYC2 and probably other JA signaling transcription factors, repressing their transcription activity. When the concentration of JA-Ile or coronatine reaches a threshold during wounding or pathogen infection (Chung *et al.*, 2008), COI1 binds to the Jas

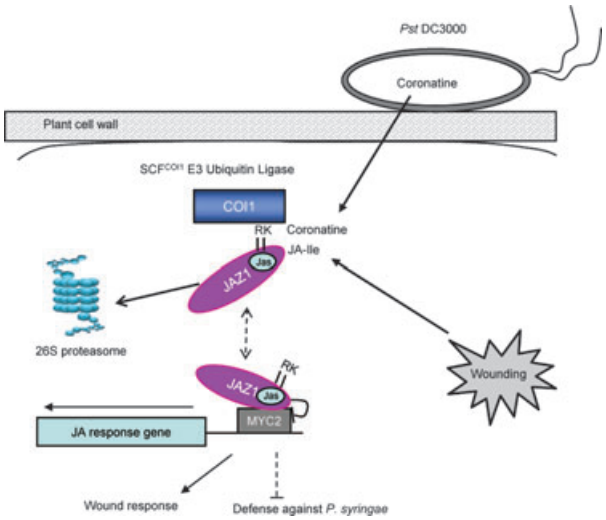

Figure 6. A model for the action of coronatine and JA-Ile in the plant cell.
In the absence of JA-Ile or coronatine, JAZ proteins bind to AtMYC2 and possibly other JA signaling transcription factors (not shown) via the C-terminal Jas domain, repressing their transcription activity. When the concentration of JA-Ile or coronatine reaches a threshold during wounding or pathogen infection, COI1 binds to the Jas domain of JAZ proteins in a ligand-dependent manner. The positively charged residues of the Jas domain (R205R206 in JAZ1 and R223K224 in JAZ9) play a prominent role in this interaction. The JA-Ile/coronatine-dependent interaction between COI1 and the C-terminus of JAZ proteins results in SCF^{COI1}-dependent ubiquitination and degradation of JAZ proteins via the 26S proteasome, thus de-repressing JA signaling, which activates the wound response and suppresses plant defense against *Pseudomonas syringae* bacteria.

domain of JAZ proteins, presumably in a ligand-dependent manner. Interestingly, whereas R205R206 in JAZ1 and R223K224 in JAZ9 are important for JAZ1–COI1 or JAZ9–COI1 interactions, they are not important in mediating JAZ1–AtMYC2 or JAZ9–AtMYC2 interactions (Figure 5), suggesting that the interacting surfaces for JAZ–COI1 and JAZ–AtMYC2 interactions are not identical. The JA-Ile/coronatine-dependent interaction between COI1 and the C-terminus of JAZ proteins results in SCF^{COI1}-dependent ubiquitination and degradation of JAZ proteins through the 26S proteasome, thus de-repressing JA signaling. This Jas domain-based model is different from a previous model proposed by Chini *et al.* (2007) based on observations on the JAZ3 interaction with COI1 and AtMYC2 proteins. In these experiments, purified recombinant JAZ3 protein was found to interact with the COI1 protein produced in a wheatgerm *in vitro* transcription/translation extract, and the COI1–JAZ3 interaction did not require exogenous jasmonates. Moreover, the N-terminal/central regions (containing the NT and ZIM domains), but not the C-terminus (containing the Jas domain), were sufficient for this interaction. Our revised model could in principle accommodate the observations by Chini *et al.* regarding the COI1–JAZ3 interaction. For example, the JA-Ile-independent NT/ZIM-domain-mediated interaction could represent a basal level interaction that is detectable only when COI1 is produced in the wheatgerm *in vitro* transcription and translation extract (Chini *et al.*, 2007)), but not in Y2H or protein pull-down assays (Thines *et al.*, 2007; Katsir *et al.*, 2008; this study). As proposed by Chini *et al.* (2007), this ligand-independent interaction between COI1 and the N-terminus of JAZ proteins may provide an explanation for the dominant-negative effect of JAZ protein derivatives that lack a functional Jas motif. Future crystal structure analyses of COI1–JAZ complexes in the presence or absence of JA-Ile/coronatine would help to distinguish between these possibilities.

Experimental procedures

Plant material and growth conditions

Arabidopsis thaliana plants were grown in soil under the light intensity of 100 μE m^{-2} sec^{-1} at 20 to 22°C. In all experiments, wild-type (WT) refers to the Columbia (Col-0) ecotype. To generate Arabidopsis Col-0 plants expressing the COI1–Myc fusion protein, the *COI1* cDNA was amplified by RT-PCR using primers COI1up (5′-TTTTGTCGACCCGATGGAGGATC-3′; *Sal*I recognition site underlined) and COI1dn1 (5′-GGGTGGTACCATATTGGCTCCTT-3′; *Kpn*I recognition site underlined), and cloned into a pCambia1300 derivative, behind the CaMV 35S promoter and in front of a 9xMyc tag (398 bp) fragment. The DNA insert was fully sequenced. The resulting plasmid (pCambia1300-COI1-9Myc) was introduced into *Agrobacterium tumefaciens* strain GV3101 (pMP90) for transformation into Arabidopsis Col-0 (*COI1*/*COI1*) and heterozygous *COI1*/*coi1-1* plants using the floral dip method (Clough and Bent, 1998). Correct functioning of the COI1–Myc fusion was confirmed by observing restoration of root sensitivity to 50 μM JA when the *coi1*/*COI1*-Myc progeny were germinated on MS agar plates and male fertility in the later stages of development (data not shown). Expression of COI1–Myc in multiple T_1 plants was confirmed by immunoblotting using the Myc epitope antibody. Homozygous T_4 Col-0 plants expressing COI1–Myc were used for the experiments.

Transgenic plants expressing a modified *JAZ1* cDNA encoding JAZ1-A205A206 were produced using the techniques and vectors described by Thines *et al.* (2007). Tomato plants expressing the functional *Sl*COI1–Myc fusion and growth conditions were as described previously (Thines *et al.*, 2007).

Protein expression constructs

The *JAZ1* cDNA was amplified by PCR using the primers JAZ1-Not1 (5′-GCGCGGCCGCCATGTCGAGTTCTATGGAATGTTCT-3′) and JAZ1-Xho1 (5′- CCCTCGGTATTTCAGCTGCTAAACCGAG-3′; restriction sites underlined). The PCR product was digested with *Not*I and *Xho*I and cloned into the corresponding sites of pRMG-nMAL (Thines *et al.*, 2007). To produce the MBP–JAZ9–6xHis fusion construct, the MBP coding sequence was released from pMAL-c4x (NEB Laboratories, http://www.neb.com) by *Nde*I and *Sal*I digestion, and sub-cloned into the same sites of pET42b (Novagen, http://www.emdbiosciences.com). The *JAZ9* cDNA was amplified by PCR using the primers JAZ9-F1 (5′-CACCGAATTCCCCATGGAAAGAGATTTTCTGGGTTTG; *Eco*RI recognition site underlined) and JAZ9-R1 (5′-TTACTCGAGGGCGCGCCCTGTAGGAGAAGTAGAAGAGTAA; *Xho*I recognition site underlined), and cloned into the *Eco*RI and *Xho*I sites of pET-MAL to create the MBP–JAZ9–6xHis fusion

construct. Fusion proteins were expressed in *Escherichia coli* strain Rosetta 2(DE3)pLysS. The recombinant proteins were purified by using amylase resin, and eluted using 10 mM maltose in column buffer (20 mM Tris/HCl pH 7.4, 200 mM NaCl, 10 mM β-mercaptoethanol).

Yeast two-hybrid (Y2H) methods

The Arabidopsis gene *COI1* (At2g39940) was cloned into the Y2H bait vector pGILDA (Clontech, http://www.clontech.com/) resulting in a LexA–COI1 protein fusion. This gene construct was transformed into yeast (*Saccharomyces cerevisae*) strain EGY48 (p8opLacZ) using the frozen-EZ yeast transformation II kit (Zymo Research, http://www.zymoresearch.com). Tranformants were selected on SD-glucose medium (BD Biosciences, http://www.bdbiosciences.com) supplemented with –Ura/–His drop-out solution (BD Biosciences). An Arabidopsis cDNA library (Holt *et al.*, 2002) was screened twice for coronatine-dependent COI1 interactors using the Matchmaker LexA two-hybrid system (Clontech) according to the manufacturer's instructions, except that coronatine (1.5 μM for one screen and 50 μM for the other) was added to the inducing medium [SD-galactose/rafinose inducing medium (BD Biosciences) containing–Ura/–His/–Trp drop-out supplement and 80 μg ml^{-1} X-Gal].

To detect the interaction between JAZ protein and COI1 or MYC2, the *JAZ* genes were amplified by RT-PCR from Arabidopsis leaves collected 12 h after inoculation with the pathogen *P. syringae* pv. *tomato* (*Pst*) DC3000. *JAZ* cDNAs were cloned into the Y2H prey vector pB42AD to obtain a B42–HA–JAZ fusion protein and co-transformed with pGILDA-COI1 into EGY48 (p8opLacZ) using the frozen-EZ yeast transformation II kit (Zymo Research). For Y2H assay with AtMYC2 and JAZ proteins, *JAZ* cDNAs were cloned into the Y2H bait vector pGILDA to obtain a LexA–JAZ fusion protein and co-transformed with pB42AD-AtMYC2 into EGY48 (p8opLacZ) using the frozen-EZ yeast transformation II kit (Zymo Research). Tranformants were selected on SD-glucose medium supplemented with –Ura/–His/–Trp drop-out solution. Expression of the LexA–COI1 and B42–HA–JAZ protein fusions was detected by Western blotting using epitope-specific antibodies. To assess the JA-Ile- or coronatine-dependent interaction between COI1 and JAZ proteins, transformed yeast strains were plated on SD-galactose/rafinose inducing medium containing –Ura/–His/–Trp drop-out supplement and 80 μg ml^{-1} X-Gal. In some experiments (as indicated in figure legends), one of the following chemicals was also added: 60 μM JA-Ile, 60 μM jasmonic acid (Sigma, http://www.sigmaaldrich.com/), 60 μM methyl jasmonate (Sigma), 60 μM OPDA (Cayman Chemical Company, http://www.caymanchem.com), 60 μM coronatine (Oklahoma State University or Sigma) or 10% ethanol (the solvent for all the chemicals). Plates were incubated for up to 6 days at 30°C. A positive control strain containing the pLexA-53 and pB42AD-T plasmids (Clontech) was also plated on inducing medium for comparison of the colony color.

Protein pull-down assay

Four-week-old Arabidopsis or 3-week-old tomato plants that stably express the COI1–Myc fusion protein were ground in liquid nitrogen to fine powder. Soluble proteins were extracted using binding buffer (50 mM Tris/HCl pH 6.8, 150 mM NaCl, 10% glycerol, 0.1% Tween-20, 20 mM imidazole, 20 mM β-mercaptoethanol, 1% Sigma protease inhibitor cocktail, and 10 mM MG-132), and clarified by centrifugation at 15 000 ***g*** at 4°C for 20 min. Protein concentrations were determined using a Bio-Rad RC DC assay kit (http://www.bio-rad.com/). Protein samples were divided into aliquots and stored at −80°C. For each pull-down assay, 1 mg of soluble protein extracts from Arabidopsis or tomato plants was incubated with 25 μg purified MBP–6xHis or MBP–JAZ–6xHis fusion protein in a final volume of 0.5 ml. The procedure for protein pull-down experiments was as described previously (Thines *et al.*, 2007).

Site-directed mutagenesis

Individual amino acid residues in the Jas domain of JAZ1 and JAZ9 proteins were mutated to alanine in pB42AD using the QuickChange II site-directed mutagenesis kit (Stratagene, http://www.stratagene.com/). Mutant proteins were co-expressed with COI1 (expressed from pGILDA::COI1) in yeast to detect protein–protein interactions, as described above. To study the effect of Jas domain mutations on JAZ–AtMYC2 interaction, we moved the mutated JAZ1 and JAZ9 inserts from pB42AD into pGILDA. Mutant JAZ1 and JAZ9 proteins were co-expressed with AtMYC2 (expressed from pB42AD::AtMYC2) in yeast to detect protein–protein interactions, as described above.

Bacterial infection assay

Pst DC3000 was cultured at 30°C in Luria–Bertani (LB) medium supplemented with appropriate antibiotics until an attenuance at 600 nm (D_{600}) of 0.8 was reached. Bacteria were collected by centrifugation (2 000 ***g*** for 30 min at 25°C) and resuspended in water to a final concentration of 10^6 colony-forming units per ml. Four-week-old Arabidopsis plants were infiltrated with bacterial suspension and kept under high humidity until disease symptoms developed. The bacterial population in the plant apoplast was determined as previously described (Katagiri *et al.*, 2002).

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Alignment of *JAZ9* cDNA clones isolated from a yeast two-hybrid screening using Arabidopsis COI1 as bait.

This material is available as part of the online article from http://www.blackwell-synergy.com.

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References

Bender, C.L., Alarcon-Chaidez, F. and Gross, D.C. (1999) *Pseudomonas syringae* phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. *Microbiol. Mol. Biol. Rev.* **63**, 266–292.

Block, A., Schmelz, E., Jones, J.B. and Klee, H.J. (2005) Coronatine and salicylic acid: the battle between *Arabidopsis* and *Pseudomonas* for phytohormone control. *Mol. Plant Pathol.* **6**, 79–83.

Brooks, D.M., Bender, C.L. and Kunkel, B.N. (2005) The *Pseudomonas syringae* phytotoxin coronatine promotes virulence by overcoming salicylic acid-dependent defences in *Arabidopsis thaliana*. *Mol. Plant Pathol.* **6**, 629–639.

Browse, J. (2005) Jasmonate: an oxylipin signal with many roles in plants. In *Vitamins and Hormones* (Litwack, G., ed.). New York: Academic Press, pp. 431–456.

Browse, J. and Howe, G.A. (2008) Update on jasmonate signaling: new weapons and a rapid response against insect attack. *Plant Physiol.*, **146**, 832–838.

Chini, A., Fonseca, S., Fernandez, G. *et al.* (2007) The JAZ family of repressors is the missing link in jasmonate signalling. *Nature*, **448**, 666–671.

Chung, H.S., Koo, A.J.K., Gao, X., Jayany, S., Thines, B., Jones, A.D. and Howe, G.A. (2008) Regulation and function of Arabidopsis JAZ genes in response to wounding and herbivory. *Plant Physiol.*, **146**, 952–964.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743.

Cui, J., Bahrami, A.K., Pringle, E.G., Hernandez-Guzman, G., Bender, C.L., Pierce, N.E. and Ausubel, F.M. (2005) *Pseudomonas syringae* manipulates systemic plant defenses against pathogens and herbivores. *Proc. Natl Acad. Sci. USA*, **102**, 1791–1796.

Feys, B., Benedetti, C.E., Penfold, C.N. and Turner, J.G. (1994) Arabidopsis mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. *Plant Cell*, **6**, 751–759.

Holt, B.F., Boyes, D.C., Ellerstrom, M., Siefers, N., Wiig, A., Kauffman, S., Grant, M.R. and Dangl, J.L. (2002) An evolutionarily conserved mediator of plant disease resistance gene function is required for normal Arabidopsis development. *Dev. Cell*, **2**, 807–817.

Howe, G.A. and Jander, G. (2008) Plant immunity to herbivores. *Annu. Rev. Plant Biol.* **59**, 41–66.

Katagiri, F., Thilmony, R. and He, S.Y. (2002) The *Arabidopsis thaliana–Pseudomonas syringae* interaction. In *The Arabidopsis Book* (Somerville, C.R. and Meyerowitz, E.M., eds). Rockville, MD: American Society of Plant Biologists, doi: 10.1199/tab.0039 (http://www.aspb.org/publications/arabidopsis/).

Katsir, L., Schilmiller, A.L., Staswick, P.E., He, S.Y. and Howe, G.A. (2008) COI1 is a critical component of a receptor for jasmonoyl-isoleucine and the bacterial virulence factor coronatine. *Proc. Natl Acad. Sci. USA*, **105**, 7100–7105.

Kloek, A.P., Verbsky, M.L., Sharma, S.B., Schoelz, J.E., Vogel, J., Klessig, D.F. and Kunkel, B.N. (2001) Resistance to *Pseudomonas syringae* conferred by an *Arabidopsis thaliana* coronatine-insensitive (*coi1*) mutation occurs through two distinct mechanisms. *Plant J.* **26**, 509–522.

Lauchli, R. and Boland, W. (2003) Indanoyl amino acid conjugates: tunable elicitors of plant secondary metabolism. *Chem. Rec.* **3**, 12–21.

Li, L., Li, C.Y., Lee, G.I. and Howe, G.A. (2002) Distinct roles for jasmonate synthesis and action in the systemic wound response of tomato. *Proc. Natl Acad. Sci. USA*, **99**, 6416–6421.

Melotto, M. and He, S.Y. (2007) AtCOI1, an E3 ubiquitin ligase subunit, interacts with putative target proteins in a coronatine/jasmonate-dependent manner. In *Book of Abstracts, XIII International Congress on Molecular Plant–Microbe Interactions, Sorrento, Italy, 21–27 July 2007*, p. 403.

Melotto, M., Underwood, W., Koczan, J., Nomura, K. and He, S.Y. (2006) Plant stomata function in innate immunity against bacterial invasion. *Cell*, **126**, 969–980.

Melotto, M., Underwood, W. and He, S.Y. (2008) Role of stomata in plant innate immunity and foliar bacterial diseases. *Annu. Rev. Phytopathol.*, **46**, 101–122.

Mittal, S.M. and Davis, K.R. (1995) Role of the phytotoxin coronatine in the infection of *Arabidopsis thaliana* by *Pseudomonas syringae* pv. *tomato*. *Mol. Plant Microbe Interact.* **8**, 165–171.

Staswick, P.E. (2008) JAZing up jasmonate signaling. *Trends Plant Sci.* **13**, 66–71.

Tan, X., Calderon-Villalobos, L.I.A., Sharon, M., Zheng, C., Robinson, C.V., Estelle, M. and Zheng, N. (2007) Mechanism of auxin perception by the TIR1 ubiquitin ligase. *Nature*, **446**, 640–645.

Thilmony, R., Underwood, W. and He, S.Y. (2006) Genome-wide transcriptional analysis of the *Arabidopsis thaliana* interaction with the plant pathogen *Pseudomonas syringae* pv. *tomato* DC3000 and the human pathogen *Escherichia coli* O157:H7. *Plant J.* **46**, 34–53.

Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., Nomura, K., He, S.Y., Howe, G.A. and Browse, J. (2007) JAZ repressor proteins are targets of the SCF^{COI1} complex during jasmonate signaling. *Nature*, **448**, 661–665.

Underwood, W., Melotto, M. and He, S.Y. (2007) Role of plant stomata in bacterial invasion. *Cell. Microbiol.* **9**, 1621–1629.

Uppalapati, S.R., Ayoubi, P., Weng, H., Palmer, D.A., Mitchell, R.E., Jones, W. and Bender, C.L. (2005) The phytotoxin coronatine and methyl jasmonate impact multiple phytohormone pathways in tomato. *Plant J.* **42**, 201–217.

Vanholme, B., Grunewald, W., Bateman, A., Kohchi, T. and Gheysen, G. (2007) The tify family previously known as ZIM. *Trends Plant Sci.* **12**, 239–244.

Xie, D.X., Feys, B.F., James, S., Nieto-Rostro, M. and Turner, J.G. (1998) *COI1*: an *Arabidopsis* gene required for jasmonate-regulated defense and fertility. *Science*, **280**, 1091–1094.

Yan, Y., Stolz, S., Chetelat, A., Reymong, P., Pagni, M., Dubugnon, L. and Farmer, E.E. (2007) A downstream mediator in the growth repression limb of the jasmonate pathway. *Plant Cell*, **19**, 2470–2483.

Zhao, Y., Thilmony, R., Bender, C.L., Schaller, A., He, S.Y. and Howe, G.A. (2003) Virulence systems of *Pseudomonas syringae* pv. tomato promote bacterial speck disease in tomato by targeting the jasmonate signaling pathway. *Plant J.* **36**, 485–499.