Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2012) **13**(1), 46–57 DOI: 10.1111/J.1364-3703.2011.00727.X

Analysis of Arabidopsis *JAZ* gene expression during *Pseudomonas syringae* pathogenesis

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SUMMARY

The jasmonates (JAs) comprise a family of plant hormones that regulate several developmental processes and mediate responses to various abiotic and biotic stresses, including pathogens. JA signalling is manipulated by several strains of the bacterial pathogen *Pseudomonas syringae*, including *P. syringae* strain DC3000, using the virulence factor coronatine (COR) as a mimic of jasmonyl-L-isoleucine (JA-Ile). To better understand the JA-Ile-mediated processes contributing to *P. syringae* disease susceptibility, it is important to investigate the regulation of JA signalling during infection. In *Arabidopsis thaliana*, JASMONATE ZIM-DOMAIN (JAZ) proteins are negative regulators of JA signalling. The transcription factor JASMONATE INSENSITIVE1 (JIN1/ATMYC2) has been implicated in the regulation of *JAZ* gene expression. To investigate the regulation of *JAZ* genes during *P. syringae* pathogenesis, we examined *JAZ* gene expression during infection of Arabidopsis by DC3000. We found that eight of the 12 *JAZ* genes are induced during infection in a COR-dependent manner. Unexpectedly, the induction of the majority of *JAZ* genes during infection was not dependent on *JIN1*, indicating that JIN1 is not the only transcription factor regulating *JAZ* genes. A T-DNA insertion mutant and an RNA interference line disrupted for the expression of *JAZ10*, one of the few *JAZ* genes regulated by *JIN1* during infection, exhibited enhanced JA sensitivity and increased susceptibility to DC3000, with the primary effect being increased disease symptom severity. Thus, *JAZ10* is a negative regulator of both JA signalling and disease symptom development.

INTRODUCTION

Plants use hormones to regulate development, growth and responses to external stimuli. Oxylipin compounds, collectively referred to as jasmonates (JAs), are an important family of plant hormones. JAs regulate aspects of development, such as root growth, stamen and pollen development, and senescence, as well as responses to environmental stresses, such as wounding, insect attack and pathogen infection (Browse, 2009; McConn and Browse, 1996; McConn *et al*., 1997; Staswick *et al*., 1998; Vijayan *et al*., 1998). Many plant pathogens have evolved virulence strategies to modulate hormone signalling in their hosts to facilitate infection and disease production (Grant and Jones, 2009). For example, several strains of the bacterial pathogen *Pseudomonas syringae*, including *P. syringae* strain DC3000, manipulate the JA signalling pathway by producing the virulence factor coronatine (COR) (Brooks *et al*., 2005; Kunkel and Chen, 2006). COR is a molecular mimic of jasmonyl-L-isoleucine (JA-Ile), the endogenous active form of JA (Fonseca *et al*., 2009; Staswick and Tiryaki, 2004), and is required for the colonization of host tissue, suppression of salicylic acid (SA)-mediated host defences and production of disease symptoms (Bender *et al*., 1998; Brooks *et al*., 2004, 2005; Melotto *et al*., 2006).

In Arabidopsis, JA signalling is initiated when JA-Ile binds to a receptor complex formed by CORONATINE INSENSITIVE1 (COI1) and a JASMONATE ZIM-DOMAIN (JAZ) protein (Katsir *et al*., 2008a, b; Sheard *et al*., 2010; Yan *et al*., 2009). COI1, initially identified in a screen for mutants exhibiting insensitivity to COR (Feys *et al*., 1994), is the F-box protein in the SCF^{COI1} ubiquitin E3 ligase complex that targets repressors of JA signalling, such as JAZ proteins, for degradation (Devoto *et al*., 2002; Katsir *et al*., 2008a; Xie *et al*., 1998). In the presence of JA-Ile, JAZ proteins interact with COI1 and are degraded, leading to the activation of JA-responsive gene expression (Chini *et al*., 2007; Thines *et al*., 2007). Although these findings uncover a mechanism whereby plants sense and respond to JA, it is still unclear how multiple JA-regulated cues are translated into highly specific responses.

The regulation of JA responses by *JAZ* proteins provides one possible mechanism for the fine-tuning of responses. There are 12 *JAZ* genes in Arabidopsis (Chini *et al*., 2007; Thines *et al*., 2007; Yan *et al*., 2007). JAZ proteins share two highly conserved motifs: the TIFY/ZIM domain important for JAZ homo- and heterodimerization, and the Jas motif which is required for JAZ–COI1 interactions and degradation in response to JA-Ile (Chini *et al*., 2009; Chung and Howe, 2009; Melotto *et al*., 2008; Thines *et al*., 2007). As JAZ proteins do not have a DNA-binding domain, it is likely that they repress JA responses indirectly, possibly by interacting with one or more transcription factors (Chini *et al*., 2007; Thines *et al*., 2007). Several dominant negative mutants encoding truncated JAZ proteins missing the Jas motif (JAZΔJas) have been characterized (Chini *et al*., 2007; Chung *et al*., 2010; Chung and Howe, 2009; Thines *et al*., 2007; Yan *et al*., 2007). JAZΔJas proteins are resistant to COI1-mediated degradation and most confer JA

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insensitivity, demonstrating the role of JAZ proteins as repressors of JA signalling. Loss-of-function *jaz* mutants, however, are predicted to exhibit enhanced responses to JA. RNA interference (RNAi) lines have been generated for *JAZ1* and *JAZ10*; the plants are hypersensitive to JA, consistent with a role for these two proteins as repressors (Grunewald *et al*., 2009; Yan *et al*., 2007). A mutant line carrying a T-DNA insertion in the *JAZ1* regulatory region has recently been reported to be hypersensitive to JA (Grunewald *et al*., 2009). Preliminary examination of several other *JAZ* insertion mutants has not identified any with JA-related phenotypes (Chini *et al*., 2007; Thines *et al*., 2007); however, a systematic examination of mutants in the gene family or of higher order mutants has not been reported.

Differential regulation of *JAZ* gene expression is another possible mechanism to provide specificity to JA responses. *JAZ* genes are rapidly induced by JA, suggesting a negative feedback loop to replenish the JAZ protein pool and to dampen the response to JA (Chini *et al*., 2007; Thines *et al*., 2007). Evidence points to a role for the transcription factor JASMONATE INSENSITIVE1 (JIN1/AtMYC2) in the regulation of *JAZ* genes. The expression of several *JAZ* genes is reduced in *jin1* mutants treated with JA, and JIN1 binds directly to the promoter region of *JAZ3* (Chini *et al*., 2007). JIN1 has also been shown to interact with most JAZ proteins *in vitro*, leading to the hypothesis that the binding of JAZs to JIN1 in the absence of JA prevents JIN1 from transcribing JA-responsive genes, including the *JAZ* genes themselves (Chini *et al*., 2007, 2009; Melotto *et al*., 2008). The role of JIN1 in JA signalling is well established. JIN1 positively regulates wound-responsive genes and negatively regulates several pathogen-responsive genes (Lorenzo *et al*., 2004; Nickstadt *et al*., 2004). Thus, given that *jin1* mutants are still partially JA responsive, there are likely to be additional transcription factors regulating JA responses and, possibly, *JAZ* gene expression as well.

Recently, transcriptional analysis of *JAZ* genes in response to herbivory and wounding has provided evidence of specificity in *JAZ* gene induction in response to these two stimuli (Chung *et al*., 2008). Specifically, *JAZ7* and *JAZ8* were induced much more robustly after mechanical wounding compared with after feeding by *Spodoptera exigua* larvae. These observations suggest that *JAZ* genes are regulated at the transcriptional level and that specificity in the JA response may be the result of their differential regulation. This finding also raises the possibility that the manipulation of *JAZ* gene expression to alter JA signalling could be a virulence strategy.

Pseudomonas syringae strain DC3000 colonizes the apoplast of tomato and Arabidopsis. Stimulation of JA-mediated responses by COR is an important component of DC3000 pathogenesis and promotes both bacterial growth and disease symptom development. DC3000 COR-deficient mutants are severely compromised for virulence (Brooks *et al*., 2004, 2005). In addition, Arabidopsis mutants impaired in JA signalling, such as *jin1*, *coi1* and several *JAZΔJas* mutants, have reduced sensitivity to COR and are less susceptible to DC3000 (Kloek *et al*., 2001; Laurie-Berry *et al*., 2006; Thines *et al*., 2007).

Defence responses to *P. syringae* are mediated by SA, and Arabidopsis mutants impaired in SA biosynthesis, such as those disrupting *SA INDUCTION DEFICIENT 2/ISOCHORISMATE SYNTHASE 1* (*SID2/ICS1*), are highly susceptible to *P. syringae* (Dewdney *et al*., 2000; Wildermuth *et al*., 2001). Regulatory crosstalk between the JA and SA signalling pathways is well established and the stimulation of JA signalling can result in the downregulation of SA-mediated defences (Brooks *et al*., 2005; Kunkel and Brooks, 2002; Spoel and Dong, 2008). *jin1* and *coi1* mutants exhibit enhanced expression of SA-mediated defences during infection (Kloek *et al*., 2001; Laurie-Berry *et al*., 2006; Nickstadt *et al*., 2004), indicating that one role of COR is to stimulate JA signalling, thereby suppressing SA-mediated defences and promoting bacterial growth. Interestingly, although bacterial growth is restored to wild-type levels in *jin1 sid2* double mutants, these plants develop only mild disease symptoms (Laurie-Berry *et al*., 2006). These results indicate that there are two branches of the JA signalling pathway manipulated by DC3000, one to suppress SA defences and a distinct pathway to enhance symptom production.

To better understand the JA-Ile-mediated processes that contribute to *P. syringae* disease susceptibility, it is important to investigate the regulation of JA signalling during infection. In this study, we investigated the regulation of *JAZ* genes during DC3000 pathogenesis and found that eight of the 12 *JAZ* genes are induced after infection and that this induction is dependent on COR. *JAZ* gene induction was independent of *JIN1* for most *JAZ* genes, indicating that JIN1 is not the sole transcription factor involved in *JAZ* regulation during infection. In addition, we showed that *JAZ10* is a negative regulator of the branch of the JA pathway required for DC3000 symptom development.

RESULTS

Most *JAZ* genes are induced during DC3000 infection

Whole-genome transcriptional analysis of Arabidopsis, 24 h after infiltration with DC3000, revealed that a subset of *JAZ* genes, *JAZ2*, *JAZ5*, *JAZ6*, *JAZ7*, *JAZ9* and *JAZ10*, is induced by DC3000 (Thilmony *et al*., 2006). *JAZ4* and *JAZ11* are not represented on Affymetrix ATH1 arrays, and so their expression during DC3000 infection is unknown. To more thoroughly investigate *JAZ* gene expression during DC3000 infection, we examined *JAZ* transcript levels at several time points after dip infection or mock infection (Fig. 1). Most *JAZ* genes were induced by mock treatment at 2–6 h, but induction subsided to basal levels by 24 h. Only *JAZ11* and *JAZ12* were unaffected by mock treatment. *JAZ4* was expressed at very low levels, consistent with previous observations (Chung *et al*., 2008). During DC3000 infection, *JAZ* genes were

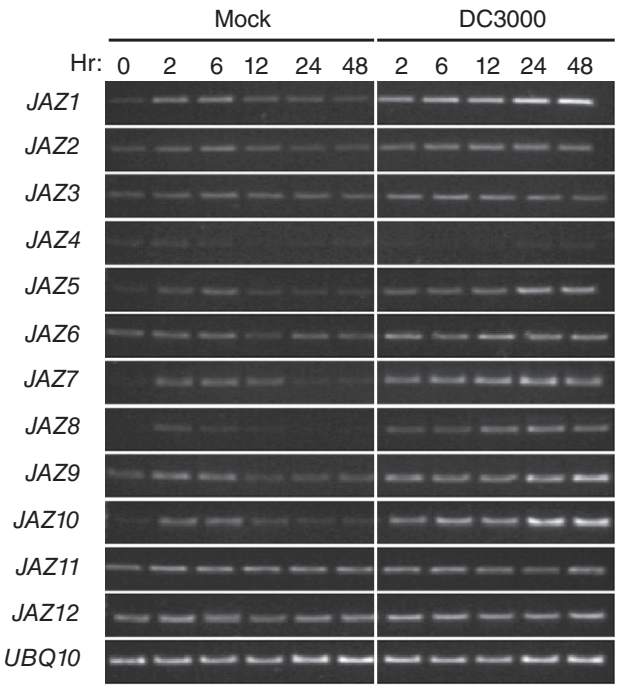

Fig. 1 *JASMONATE ZIM-DOMAIN* (*JAZ*) gene expression during DC3000 infection. Arabidopsis (Col-0) plants were dip inoculated in DC3000 or $MgCl_2$ for mock inoculation. Untreated plants were used for the 0-h time point. Transcript levels were monitored by reverse transcription-polymerase chain reaction (RT-PCR) in RNA samples isolated from tissue harvested at the indicated times (hours post-infection) after inoculation. *UBQ10* (At4g05320) is shown as an internal control.

induced similarly to mock treatment at early time points. However, for most genes, expression remained high 12–48 h after infection (Fig. 1). All *JAZ* genes, except *JAZ3*, *JAZ4*, *JAZ11* and *JAZ12*, were induced by DC3000 infection. Overall, our data suggest that the expression of *JAZ* genes at 24 and 48 h after infection is a specific response to DC3000 and not a result of wounding or other abiotic responses related to the treatment.

Induction of *JAZ* genes during infection is dependent on COR

It is likely that the induction of *JAZ* genes during infection is caused, at least in part, by the activity of COR, as it is a mimic of JA-Ile. However, it is possible that other virulence factors could contribute to the induction of *JAZ* genes. To determine the extent to which the induction of *JAZ* genes during DC3000 infection is dependent on COR, we examined the expression of *JAZ* genes after inoculation with wild-type DC3000 or a DC3000 COR biosynthetic mutant (COR^-) (Brooks *et al*., 2004). We used real-time reverse transcription-polymerase chain reaction (RT-PCR) to discern small changes in transcript levels, focusing on the 24- and 48-h time points, as responses to mock treatment have diminished by this time. All *JAZ* genes induced during DC3000 infection in the previous experiment (Fig. 1) were also induced in these experiments (Fig. 2). *JAZ4* and *JAZ11* were not induced significantly, whereas *JAZ3* and *JAZ12* were minimally induced in some, but not all, experiments (for example, see Fig. 3); therefore, it is unlikely that the induction of these genes is solely a result of DC3000 infection.

The induction of *JAZ* genes during infection is strongly dependent on COR. During infection with the DC3000 COR^- strain, the level of *JAZ* gene transcripts remained similar to mock treatment for all *JAZ* genes (Fig. 2). We also examined the expression of several *JAZ* genes 72 h after infection, but did not observe any additional increase in expression in plants infected with the COR^- strain, whereas the expression in wild-type infections remained elevated (data not shown). The DC3000 COR^- strain grows to essentially wild-type levels early during infection (Fig. S1; (Brooks *et al*., 2004), and so it is unlikely that the lack of *JAZ* gene induction by the COR^- strain is a result of differences in bacterial growth. These results demonstrate that COR is the main virulence factor affecting JA signalling during DC3000 infection.

Induction of *JAZ* genes during infection is not dependent on *JIN1*

To determine whether the induction of *JAZ* genes during DC3000 infection is dependent on *JIN1*, we examined *JAZ* transcript levels by real-time RT-PCR, 24 and 48 h after DC3000 treatment, in wild-type and *jin1-1* mutant plants (Fig. 3, Fig. S2). Surprisingly, unlike during infection with DC3000 COR^-, *JAZ* genes were induced in *jin1* plants. We found that no *JAZ* gene was entirely dependent on *JIN1*. Indeed, only *JAZ5* reproducibly exhibited reduced expression at both time points during infection in *jin1* mutants. A second gene, *JAZ10*, exhibited partial *JIN1* dependence in three of four experiments. For example, in the experiment shown in Fig. 3, we observed *JIN1*-independent expression of *JAZ10* but, in other experiments (Fig. S2), *JAZ10* expression was partially dependent on *JIN1*. The remainder of the genes that were induced in wild-type plants during infection were similarly induced in *jin1* plants, although there was some variability from experiment to experiment.

The variability observed in *JAZ* gene expression in our experiments is probably a result of responses to multiple environmental stimuli that varied in our experimental conditions, and also possibly caused by differences in bacterial growth. The fact that the expression level of several *JAZ* genes was reduced at 48 h in *jin1* mutants compared with the wild-type could be caused, in part, by lower levels of bacterial growth in *jin1* mutants at this time point (Fig. S1; Laurie-Berry *et al*., 2006; Nickstadt *et al*., 2004). At the same time, it is important to note that, despite the reduced bacterial growth observed in *jin1* mutants, *JAZ* gene induction was not impaired significantly.

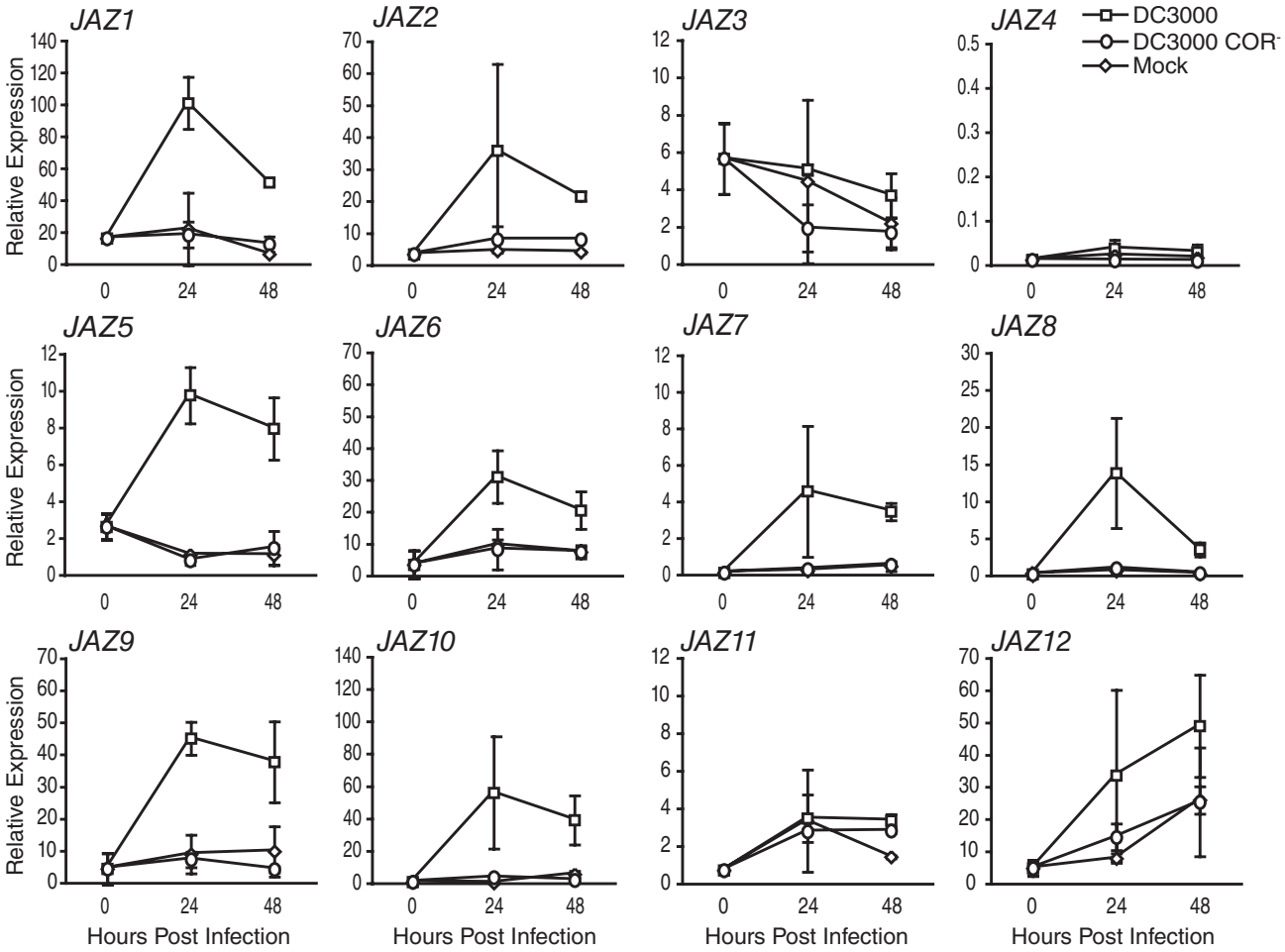

Fig. 2 Induction of *JASMONATE ZIM-DOMAIN* (*JAZ*) genes during infection is dependent on coronatine (COR). Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis of *JAZ* gene expression in wild-type plants after dip infection with DC3000 (squares), the DC3000 COR$^-$ mutant DB29 (circles) or $MgCl_2$ for mock infection (diamonds). Untreated plants were used for the 0-h time point. Gene expression at each time-point is represented relative to the internal control *PP2AA3* (At1g13320; note different scales). Error bars represent ±SD of two biological replicates. Bacterial growth over the course of infection for this experiment is shown in Fig. S1a. Similar results were obtained in a second independent experiment.

As *jin1-1* is probably a null allele (Lorenzo *et al*., 2004), our results indicate that the induction of *JAZ* genes is not dependent on JIN1, and thus one or more additional unknown transcription factors regulate *JAZ* gene expression during infection. We also observed that, in untreated plants, the basal expression of several *JAZ* genes was reduced in *jin1* mutants compared with the wild-type (data not shown), suggesting that *JIN1* plays a role in the basal expression of *JAZ* genes. Our results also do not rule out a primary role for JIN1 in the regulation of *JAZ* genes in other JA-mediated responses.

Identification and characterization of T-DNA insertional mutants in *JAZ1*, *JAZ5* and *JAZ10*

The induction of many *JAZ* genes during DC3000 infection suggests that one or more are involved in the regulation of responses to *P. syringae*. To investigate the roles of *JAZ* genes in disease susceptibility, we assayed the responses of mutants in several *JAZ* genes hypothesized to be likely to be involved in pathogenesis based on our expression data. In particular, we were interested in two genes, *JAZ5* and *JAZ10*, as these genes exhibited partial *JIN1* dependence in all or some experiments (Figs 3 and S2, and data not shown). For comparison, we also included *JAZ1*, a highly induced gene whose expression is independent of *JIN1*.

We obtained a T-DNA insertional mutant of *JAZ10* from the SAIL collection (Sessions *et al*., 2002), a T-DNA insertional mutant of *JAZ1* from the SALK collection (Alonso *et al*., 2003) (Fig. 4a) and a previously described mutation in *JAZ5*, *jaz5-1* (Thines *et al*., 2007). The mutants in *JAZ1* and *JAZ10*, designated *jaz1-1* and *jaz10-1*, accumulated no transcript, and thus are likely to be null mutants (Fig. 4b). However, we detected a transcript in *jaz5-1*, indicating that it may not be a loss-of-function mutant (Fig. 4b).

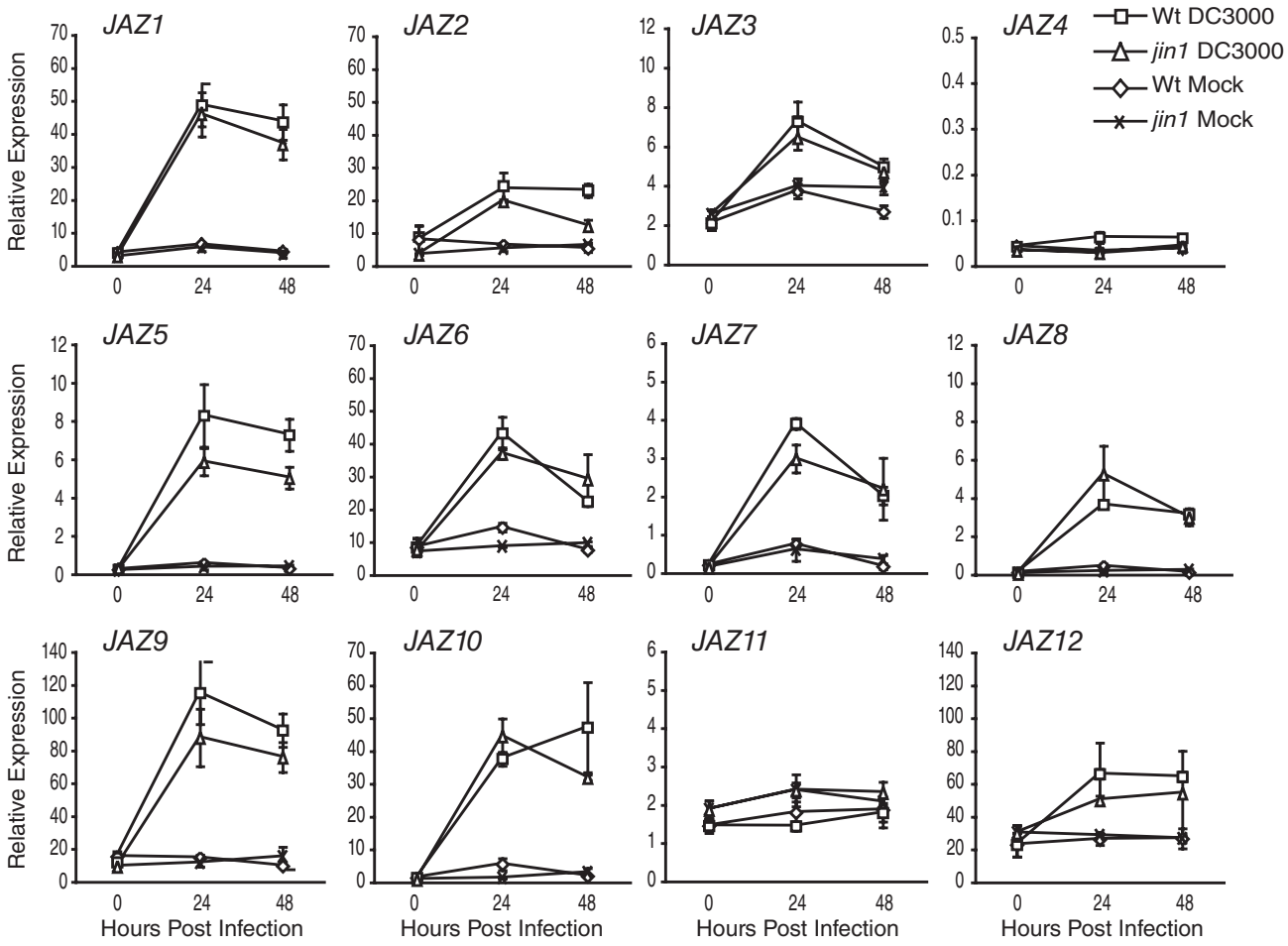

Fig. 3 *JASMONATE ZIM-DOMAIN* (*JAZ*) gene expression in *jin1* mutant plants during DC3000 infection. Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis of *JAZ* gene expression during DC3000 infection in wild-type (squares) and *jin1-1* (triangles) plants, and mock ($MgCl_2$) inoculation of wild-type (diamonds) and *jin1-1* (×) plants. Experiments were performed and analysed as described for Fig. 2. Error bars represent ±SE of three biological replicates. Bacterial growth over the course of infection for this experiment is shown in Fig. S1b. Similar results were obtained in three additional independent experiments, one of which is shown in Fig. S2.

RNAi has been used previously to generate plants with reduced *JAZ1* or *JAZ10* levels, and these plants exhibited enhanced sensitivity to methyl jasmonate (MeJA) (Grunewald *et al*., 2009; Yan *et al*., 2007). We assayed *jaz1-1* and *jaz10-1* for altered JA sensitivity in seedling root inhibition assays (Fig. 4c,d). We found that *jaz1-1* plants were essentially identical to the wild-type at all MeJA concentrations tested, including 0 μM (Fig. 4c). In previous experiments, two lines with reduced *JAZ1* expression were reported to be hypersensitive to MeJA. However, in these experiments, the mutant plants also appeared to have shorter roots when grown on medium lacking MeJA (Grunewald *et al*., 2009); thus, it is not clear whether these plants are actually hypersensitive to MeJA.

In contrast with *jaz1*-1, we found that *jaz10-1* was hypersensitive to JA (Fig. 4d) as, at every concentration tested, root growth in *jaz10-1* plants was inhibited to a greater extent than that of wild-type plants. *jaz10-1* behaved virtually identically in this assay to the *JAZ10* RNAi line, confirming that the RNAi line disrupts *JAZ10* function. Furthermore, this confirms that JAZ10 is a negative regulator of JA signalling (Chung and Howe, 2009; Yan *et al*., 2007). We did not observe any alteration in seedling root growth in the presence of MeJA in *jaz5-1* mutants (data not shown).

jaz10 mutants exhibit enhanced susceptibility to DC3000

Arabidopsis mutants with reduced sensitivity to JA and COR exhibit reduced susceptibility to *P. syringae* (Feys *et al*., 1994; Kloek *et al*., 2001; Laurie-Berry *et al*., 2006). Thus, we hypothesized that the JA-hypersensitive *jaz10-1* mutant would exhibit enhanced susceptibility to DC3000 infection. To test this, we used syringe infiltration with a low level of DC3000 to infect the

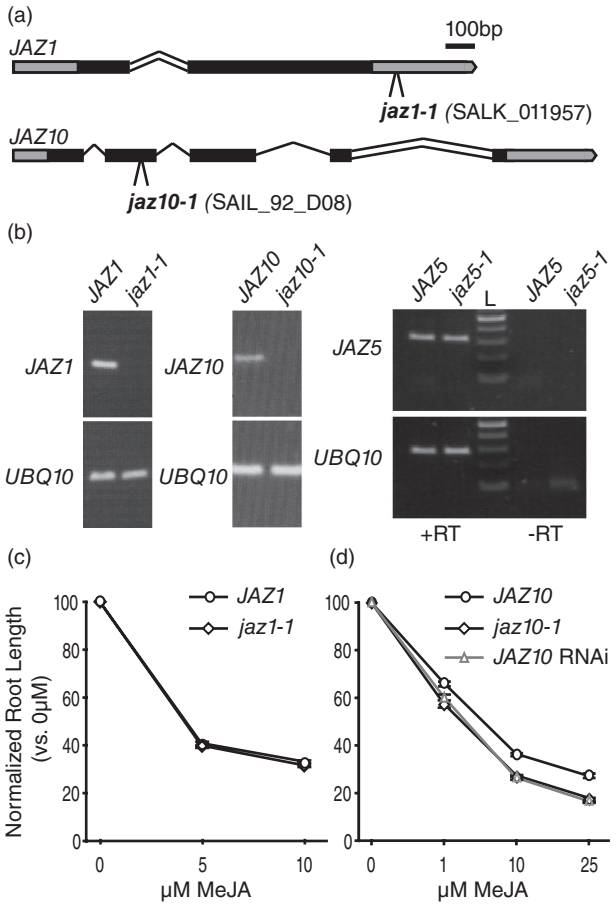

Fig. 4 Characterization of *jaz1*, *jaz5* and *jaz10* mutants. (a) Schematic diagrams of the *JAZ1* (At1g19180) and *JAZ10* (At5g13220) loci. Bars represent exons with untranslated regions in grey. Lines represent introns, with double lines indicating introns that are retained in some splice variants. The T-DNA insertion in *jaz1-1* is approximately 80 bp downstream of the end of the translated region of the gene. The T-DNA insertion in *jaz10-1* is located 20 bp from the 3′ end of the second exon. (b) Reverse transcription-polymerase chain reaction (RT-PCR) of the indicated transcripts in wild-type and *jaz* mutant plants. Transcript levels were monitored in seedlings after 10 days of growth on 10 μM methyl jasmonate (MeJA). *UBQ10* (At4g05320) was used as an internal control. For *JAZ5*, a minus reverse transcriptase (–RT) control was included to demonstrate that the PCR product corresponds to *JAZ5* transcript, not genomic DNA. (c) Jasmonate (JA) root growth inhibition assay of wild-type (circles) and *jaz1-1* (diamonds) seedlings using 0, 5 and 10 μM MeJA. The normalized root length of at least 34 plants per treatment was calculated as a percentage of the average root length of plants grown without MeJA. Error bars represent ±SE. Experiments were performed three times with similar results. (d) JA root growth inhibition assay of wild-type (circles), *jaz10-1* (diamonds) and *JAZ10* RNAi (triangles) seedlings at increasing concentrations of MeJA. The normalized root length of at least 28 plants was calculated as described in (c).

wild-type, a *JAZ10* RNAi line and *jaz10-1* mutant plants. We included *sid2-2* mutant plants as a control for enhanced susceptibility (Wildermuth *et al*., 2001). Four days after infection, many leaves of wild-type plants had no disease symptoms, and those

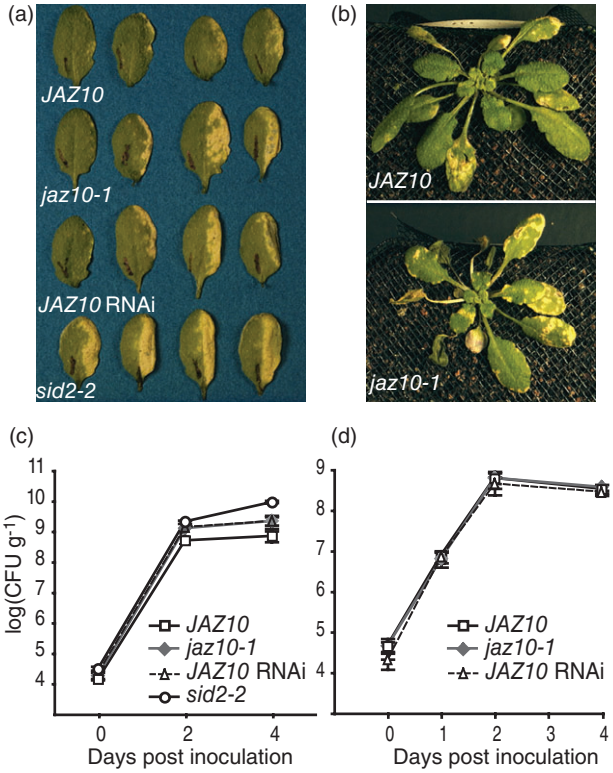

Fig. 5 *jaz10-1* is more susceptible to *Pseudomonas syringae* infection. (a) Disease symptoms of wild-type (*JAZ10*), *jaz10* lines and *sid2* leaves, 4 days after hand infiltration. The right halves (to mid-vein) of the leaves of each genotype were infiltrated with DC3000 at 5×10^5 colony-forming units (CFU)/mL. Leaves shown represent the range of symptoms exhibited by each genotype, from the mildest on the left to the most severe on the right. The black marks on the leaves are from a pen used to mark treated leaves at the time of infection. (b) Representative wild-type and *jaz10-1* mutant plants, 4 days after dip inoculation with DC3000. (c) Growth of DC3000 in wild-type (squares), *jaz10-1* (grey diamonds), *JAZ10* RNAi-9 (triangles) and *sid2-2* (circles) plants after hand infiltration. Bacteria were isolated from leaves at the indicated times after inoculation. Data points represent the average of four samples ± SE. Similar results were obtained in two additional independent experiments. (d) Growth of DC3000 in wild-type (squares), *jaz10-1* (grey diamonds) and *JAZ10* RNAi-9 (triangles) after dip inoculation. Data were collected and analysed as in (c). Similar results were obtained in two additional independent experiments.

that did develop symptoms exhibited mild to moderate chlorosis over the infiltrated area (Fig. 5a). In contrast, *JAZ10* RNAi, *jaz10-1* and *sid2* mutants exhibited significantly more severe disease symptoms than the wild-type, although *jaz10* mutant symptoms were not as severe as *sid2* (Fig. 5a). We quantified disease symptoms in each genotype by examining each infiltrated leaf to assess the percentage of the infiltrated area showing chlorosis (Table 1). Although over 50% of wild-type leaves showed no symptoms or less than 10% chlorosis over the infiltrated area, very few (approximately 12%) *jaz10-1* or *JAZ10* RNAi leaves exhibited these mild symptoms. Leaves of both *jaz10* lines generally devel-

Table 1 Quantification of disease symptoms 4 days after hand inoculation.

Symptom severity	*Arabidopsis* genotype, number of leaves (% of total)			
	Wild-type	*jaz10-1*	*JAZ10* RNAi-9	*sid2-2*
No symptoms	14 (11.1)	2 (1.7)	2 (1.9)	0 (0)
<10% chlorosis	51 (40.5)	12 (10.3)	12 (11.3)	0 (0)
10%–50% chlorosis	59 (46.8)	71 (60.7)	60 (56.6)	0 (0)
50%–100% chlorosis	2 (1.6)	31 (26.5)	30 (28.3)	65 (64.4)
100% chlorosis + collapse	0 (0)	1 (0.9)	2 (1.9)	44 (43.6)
Total leaves examined	126	117	106	101

Symptoms were quantified 4 days after infiltration as described for Fig. 5. Leaves with no symptoms were indistinguishable from uninfected leaves. Leaves with disease symptoms were grouped according to the amount of infiltrated area showing chlorosis. Similar results were seen in an independent experiment.

oped more extensive chlorosis than did wild-type leaves. As expected, leaves of *sid2* plants showed the most severe symptoms, with the majority of infected leaves having over 50% of the infiltrated area showing chlorosis.

We also observed that *jaz10-1* mutants exhibited enhanced symptom production after dip infection (Fig. 5b). Four days after infection, *jaz10-1* plants developed chlorosis and disease lesions on more leaves than wild-type plants. In addition, in *jaz10-1* plants, older leaves occasionally underwent complete collapse, a response rarely observed in wild-type plants.

We measured bacterial growth over the course of infection in both the infiltration (Fig. 5c) and dip inoculation (Fig. 5d) experiments, and found that bacterial levels in the wild-type, *jaz10-1* and *JAZ10* RNAi line were not statistically significantly different at any time point (Fig. 5c,d). The *sid2* mutant supported higher bacterial growth than both *jaz10* lines and the wild-type plants (Fig. 5c). Our results show that *JAZ10* is involved in a branch of the JA signalling pathway targeted by DC3000 to enhance susceptibility. Interestingly, defects in *JAZ10* have a more pronounced effect on symptom production than on bacterial growth.

We also used hand inoculation to assay *jaz1-1* and *jaz5-1* mutant lines for altered disease susceptibility. Bacterial growth and symptom production were similar to the wild-type in both mutants (data not shown). However, as *jaz5-1* may not be a loss-of-function mutant (Fig. 4b), we cannot conclude that *JAZ5* is not required for wild-type levels of susceptibility.

DISCUSSION

JAZ genes are differentially regulated in response to different stimuli

Our results, together with those of several other studies, suggest that JA-mediated responses to different environmental stimuli involve the differential regulation of *JAZ* genes. The expression of *JAZ* genes has been examined in only a few conditions to date. Microarray experiments examining expression at a single time point have shown that the 10 *JAZ* genes represented on the Affymetrix full genome chip are induced by MeJA treatment (Chini *et al*., 2007; Dombrecht *et al*., 2007; Thines *et al*., 2007) and six are induced by 24 h post-infection with DC3000 (Thilmony *et al*., 2006). An additional study, examining responses to wounding and herbivory by *S. exigua*, examined *JAZ* expression at multiple time points, and found that 11 *JAZ* genes are induced by mechanical wounding and herbivory (Chung *et al*., 2008). We examined *JAZ* gene expression at several time points during infection with DC3000 and found that eight of 12 *JAZ* genes are reproducibly induced by infection. Six of these have been observed previously to be induced by DC3000 (Thilmony *et al*., 2006). Differences in inoculation techniques (dip infection vs. infiltration) may account for the observed induction of the additional *JAZ* genes in our study. Our experiments also show that *JAZ* induction in response to DC3000 is distinct from the response to mock treatment. All *JAZ* genes, except *JAZ11* and *JAZ12*, were induced quickly after mock treatment and returned to untreated levels by 24 h (Fig. 1). This induction is probably a result of wounding and other stresses that may occur in the inoculation process, and is consistent with a previous study showing that wounding rapidly induces all *JAZ* genes, except *JAZ11* (Chung *et al*., 2008). It is likely that endogenous JA signalling accounts for this early induction. During DC3000 infection, *JAZ* expression remained induced 24 and 48 h after infection. As a result of the rapid initial stress response, it is difficult to determine exactly when pathogen-induced expression begins, but it is likely that pathogen-derived signals, such as COR, continue to stimulate JA signalling after the initial stress signal subsides.

Qualitatively, the pattern of *JAZ* gene expression in response to DC000 infection is similar to that observed in response to herbivory (Chung *et al*., 2008). However, there are also some notable differences in the level of gene induction for a few specific genes. For example, *JAZ7* and *JAZ8* were robustly induced in response to DC3000 (Figs 1–3), whereas these genes were only weakly induced after *S. exigua* feeding (Chung *et al*., 2008). In addition, although *JAZ10* was only moderately induced after herbivory, *JAZ10* was one of the most highly induced genes in our experiments. Thus, although both responses are mediated via JA signalling, there are also unique regulatory events governing the expression of specific *JAZ* genes in response to different stimuli. All

of these results highlight the need for further studies of *JAZ* gene expression in response to different stimuli and for the identification of the signalling components responsible for the differential regulation of *JAZ* genes.

COR is the primary virulence factor responsible for the induction of *JAZ* genes during DC3000 infection

To test whether COR is responsible for the induction of *JAZ* genes, we examined *JAZ* gene expression during infection with a DC3000 COR^- mutant. We found that *JAZ* gene induction was essentially eliminated on infection with DC3000 COR^- (Fig. 2), indicating that COR is the primary virulence factor stimulating JA signalling during DC3000 infection. However, there is evidence from other studies that additional virulence factors also modulate JA signalling. For example, AvrB, a type III secreted effector protein, induced a *COI1*-dependent gene, *RAP2.6*, in the absence of COR (He *et al*., 2004). It is possible that other *P. syringae* virulence factors have an impact on JA signalling without affecting the expression of *JAZ* genes.

JIN1 is not the only transcription factor regulating *JAZ* gene expression

Currently, JIN1 is the only transcription factor implicated in the JA-mediated induction of *JAZ* gene expression (Chini *et al*., 2007, 2009; Chung and Howe, 2009). We investigated the contribution of *JIN1* to the expression of *JAZ* genes in response to DC3000 infection. Surprisingly, we found that, for most DC3000-responsive *JAZ* genes, induction during infection was independent of JIN1 (Fig. 3 and S2).

Previous studies implicating JIN1 in *JAZ* induction examined expression in seedlings in response to JA, and found that only *JAZ6* and *JAZ8* appeared to be induced by JA independently of JIN1 (Chini *et al*., 2007). Expression of the other *JAZ* genes examined was reduced in *jin1* plants compared with the wild-type. As the previous experiment only examined expression at one time point after JA treatment, it is impossible to obtain any information about the kinetics of *JAZ* gene expression. Thus, it is possible that other *JAZ* genes were induced in *jin1* mutants, but that their expression reached wild-type levels at a time point not included in the study. Alternatively, this could provide additional evidence of the differential regulation of *JAZ* gene expression in response to different stimuli. *JAZ* gene induction during infection could be mediated by other transcription factors, or even other signalling pathways, than those responsible for the regulation of *JAZ* gene expression in seedlings treated with MeJA. In support of this, *ARF6* and *ARF8* have been implicated recently in the mediation of *JAZ1* induction by auxin (Grunewald *et al*., 2009). Thus, the expression of *JAZ* genes in response to specific stimuli is not regulated solely by JIN1. Rather, it is likely that *JAZ* genes are regulated by a combination of transcription factors, and that JIN1 is required for *JAZ* expression in certain conditions, but other transcription factors are important in others. Based on our observations, it is likely that any role played by JIN1 in the regulation of *JAZ* genes during *P. syringae* infection is in association with, or secondary to, one or more additional factors.

Transcription factors from several gene families have been implicated in JA signalling (Wasternack, 2007), and it is likely that these also regulate *JAZ* gene expression and may be targets of repression by JAZ proteins. Currently, JAZ proteins are believed to repress transcription factor activity via protein–protein interactions (Chico *et al*., 2008). The majority of JAZ proteins interact with JIN1 *in vitro* (Chini *et al*., 2007, 2009; Melotto *et al*., 2008) but, as this has not been confirmed *in planta*, the role of these interactions in regulating JA-mediated responses is unclear, and it is probable that JAZ proteins also interact with other transcription factors. As JIN1 has been shown to act with the AtMYB2 transcription factor to regulate abscisic acid (ABA)-responsive gene expression (Abe *et al*., 2003), it is tempting to speculate that JIN1 may act with MYB proteins to regulate responses to JA. In addition, processes that are dependent on COI1, but independent of JIN1, such as pollen development and wounding-induced trichome development (Mandaokar and Browse, 2009; Yoshida *et al*., 2009), may involve additional transcription factors that also regulate *JAZ* genes. It will be important to identify the additional transcription factors regulating *JAZ* gene expression to fully understand the mechanism by which JAZ proteins act as repressors of JA signalling.

JAZ10 is a negative regulator of JA signalling and disease

JIN1 is required for full susceptibility to *P. syringae* (Laurie-Berry *et al*., 2006; Nickstadt *et al*., 2004). In examining *JAZ* gene expression during DC3000 infection in *jin1* mutants, we observed that *JAZ5* and *JAZ10* transcript levels were reduced compared with those in wild-type plants, implicating these genes in the regulation of JA-mediated responses to DC3000. No phenotype was observed in *jaz1-1* and *jaz5-1* mutant plants, which do not exhibit altered JA sensitivity in seedlings (Fig. 4), although a role for *JAZ5* in *P. syringae* pathogenesis cannot be ruled out as the allele examined is not a null. However, the *jaz10-1* T-DNA mutant identified in this study, like *JAZ10* RNAi lines (Yan *et al*., 2007), is both hypersensitive to MeJA in root growth inhibition assays and develops more severe disease symptoms on infection with DC3000 (Fig. 5). Thus, *JAZ10* is a negative regulator of JA signalling in seedlings and of disease susceptibility to DC3000. Interestingly, although symptom production was clearly enhanced in the *JAZ10* RNAi and *jaz10-1* mutants, bacterial growth was not affected significantly. There is evidence that the branch of the JA signalling pathway regulating symptom production is distinct from the branch regu-

lating bacterial growth (Laurie-Berry *et al*., 2006); therefore, the *jaz10* phenotype could be a result of the hyperactivation of JA responses that contribute specifically to symptom production. The identification of the genes that are upregulated in this mutant during infection may provide clues to the mechanisms responsible for the production of disease symptoms.

The enhanced symptom production in *JAZ10* RNAi and *jaz10-1* plants could reflect an overall enhancement of JA signalling in these lines. Several transcripts can be produced from *JAZ10*, including *JAZ10.4*, a splice variant missing the important Jas motif (Chung *et al*., 2010; Chung and Howe, 2009). This truncated JAZ10 protein does not interact with COI1, is resistant to degradation in the presence of JA, but can still interact with JIN1 and several JAZ proteins. It has been proposed that, as JAZ10.4 is not degraded in the presence of JA, it can accumulate in stimulated cells, and dampen JA responses after JA signalling has been initiated (Chung and Howe, 2009). This model provides an explanation for the hypersensitive phenotype that appears to be specific to *jaz10-1* mutants (Thines *et al*., 2007; Yan *et al*., 2007). Plants overexpressing a genomic clone of *JAZ10* exhibited reduced sensitivity to MeJA, suggesting an important regulatory role for JAZ10.4 (Chung *et al*., 2010). It is still unclear whether JAZ10.4 acts systemically or is tissue specific. It is also possible that JAZ10.4 is only produced during a subset of JA-mediated responses.

Future prospects

The results of our study, taken in the context of recent studies on wounding and herbivory, illustrate that *JAZ* genes are differentially regulated in response to various environmental stresses. Likewise, certain *JAZ* genes may be developmentally regulated or show tissue-specific expression. The examination of *JAZ* gene expression in these contexts, as well as in response to additional stresses, such as ozone or fungal infection, may reveal additional layers of regulation. It is still unclear how differences in *JAZ* gene expression patterns relate to JAZ protein function. Current models of JA signalling hypothesize that *JAZ* genes are induced in response to JA in order to replenish the pool of JAZ proteins degraded by SCF^{COI1} and thus to attenuate signalling (Chico *et al*., 2008). A reasonable hypothesis based on this model is that the induction of a particular *JAZ* gene reflects the degradation of that particular protein. This has not been demonstrated experimentally but, if this is the case, the induction of a particular *JAZ* gene in response to a specific stimulus implicates that protein in the regulation of responses to that stimulus. In all studies to date, overlapping sets of *JAZ* genes were induced in response to various conditions. This suggests that individual JAZ proteins are involved in multiple signalling events, and that the various JA-mediated responses probably result from the combined activity of several JAZ proteins and other transcriptional regulators.

Loss-of-function mutants can be used to address important questions regarding the role of JAZ proteins in JA signalling. Mutants in several *JAZ* genes have been examined but, to date, JA-related phenotypes have been reported only for *jaz1* and *jaz10* mutants (Chini *et al*., 2007; Grunewald *et al*., 2009; Thines *et al*., 2007). Several *JAZ* genes are the result of genomic duplication events during the evolution of Arabidopsis (Vanholme *et al*., 2007). This fact and the extensive co-regulation of *JAZ* genes suggest that there is redundancy in this family (Chico *et al*., 2008; Thines *et al*., 2007). It is worthwhile to examine double mutants, and perhaps higher order mutants, of various *JAZ* genes to identify roles of specific *JAZ* genes in JA signalling. The regulation of responses to JA is likely to be complex and may include interactions among JAZ proteins, interactions between JAZ proteins and other transcription factors, and the regulation of *JAZ* gene expression by several transcription factors. In addition, the possibility of multiple active JA family members has not yet been excluded as a means of response modulation (Ribot *et al*., 2008; Stintzi *et al*., 2001; Wasternack, 2007). Multiple avenues of research will be necessary to unravel this web of regulatory interactions and functional redundancy.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

All Arabidopsis lines used in this study were in the Columbia (Col-0) background. The *jin1-1* mutant (Berger *et al*., 1996) was chosen because no full-length transcript is present in this line (Lorenzo *et al*., 2004). *jaz5-1* (SALK_053775) (Thines *et al*., 2007) was obtained from John Browse (Washington State University, Pullman, WA, USA). SAIL_92_D08, a T-DNA insertional mutant in *JAZ10* (At5g13220) (Sessions *et al*., 2002), and SALK_011957, a T-DNA insertional mutant in *JAZ1* (At1g19180) (Alonso *et al*., 2003), designated *jaz10-1* and *jaz1-1*, respectively, were obtained from the Arabidopsis Biological Resource Center (ABRC) (http://abrc.osu.edu). *jaz1-1* was backcrossed to wild-type Col-0 once, *jaz5-1* was backcrossed to the wild-type twice and *jaz10-1* was backcrossed three times, and a homozygous line for each mutant was chosen for further characterization. For all experiments involving these mutants, a wild-type sibling identified from the same F_2 population was used as the wild-type control. The *JAZ10* RNAi-9 line (Yan *et al*., 2007) was obtained from Edward Farmer (Univeristé de Lausanne, Lausanne, Switzerland). *sid2-2* (*eds16*) (Dewdney *et al*., 2000; Wildermuth *et al*., 2001) was obtained from Mary Wildermuth (University of California at Berkeley, Berkeley, CA, USA). Plants used for infection assays were grown in soil in growth chambers with an 8-h photoperiod at 22 °C and 75% humidity. Plants used for root inhibition assays were grown on one-half strength Murashige and Skoog (0.5 × MS) (Murashige and Skoog, 1962) plates with a 16-h photoperiod.

Bacterial strains and infection experiments

Pseudomonas syringae pv. *tomato* strain DC3000 (Cuppels, 1986) and DC3000 DB29 *cmaA cfa6* double mutant (COR^-) strain (Brooks *et al*.,

2004) were used in the infection experiments. Bacteria were grown on King's B medium (King *et al*., 1954) or nutrient–yeast extract–glucose (NYG) (Daniels *et al*., 1988) containing rifampicin at 100 μg/mL at 28 °C. For dip inoculation, whole rosette leaves of 4-week-old plants were immersed in 10 mM $MgCl_2$ with suspensions of approximately 4×10^8 colony-forming units (CFU)/mL bacteria and 0.02% (v/v) Silwet L-77 (OSi Specialties Inc., Danbury, CT, USA). Mock inoculations used 10 mM $MgCl_2$ and 0.02% Silwet L-77. Plants were kept in growth chambers under clear plastic domes, except untreated samples, for the first 24 h after treatment. For hand inoculations, bacterial suspensions of 5×10^5 CFU/mL or 1×10^6 CFU/mL (as indicated) were made in 10 mM $MgCl_2$ and syringe infiltrated into one half of fully expanded leaves of 4-week-old plants. Bacterial growth in plant tissue after dip infection or infiltration was monitored as described previously (Laurie-Berry *et al*., 2006).

MeJA root inhibition assays

Sterilized seeds were germinated and grown vertically in square plates with 0.5 × MS, pH 6.0, 1% (w/v) agar, 1% (w/v) sucrose and various concentrations (0, 1, 5, 10 and 25 μM) of MeJA (Sigma Aldrich, St. Louis, MO, USA), as described previously (Laurie-Berry *et al*., 2006). After 10 days of growth, root length was measured using NIH ImageJ (Abramoff *et al*., 2004).

Identification of *jaz1-1* and *jaz10-1*

To identify plants containing a T-DNA insertion in *JAZ1*, seedlings of the SALK_011957 line were genotyped by PCR using the LBa1 (5′-TGGTTCACGTAGTGGGCCATCG-3′) (Alonso *et al*., 2003) primer and gene-specific primers (Table S1). Individual plants homozygous for the insertion were chosen for further characterization. To identify plants containing a T-DNA insertion in *JAZ10*, seedlings of the SAIL_92_D08 line were analysed by PCR for the presence of the T-DNA using the LB1 (5′-GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC-3′) (Sessions *et al*., 2002) primer and gene-specific primers (Table S1). In lines containing the T-DNA insertion in *JAZ10*, gene-specific primers generated a slightly larger PCR product than the wild-type (Fig. S3). Plants containing both the larger PCR product and the T-DNA-specific product were crossed to the wild-type three successive times and the final F_2 population was plated on 0.5 × MS plates with 10 μM MeJA. Several plants with either wild-type root length or short roots were allowed to self-pollinate, and self-seed was plated on 0.5 × MS plates with 10 μM MeJA to identify homozygous T-DNA insertion or wild-type siblings. These plants were examined for the presence or absence of the *JAZ10* transcript (Table S1). Only plants that had short roots on MeJA, the larger gene-specific PCR product and a T-DNA-specific product lacked *JAZ10* transcript. The larger gene-specific band was sequenced, and the presence of a short, approximately 90-bp, insertion in the second exon of *JAZ10* was confirmed.

RNA extraction and gene expression

To extract RNA, all above-ground tissue from two to six adult plants was frozen in liquid nitrogen, ground to a powder and mixed with extraction buffer [250 mM Tris/HCl, pH 8.5, 375 mM NaCl, 25 mM ethylenediaminetetraacetic acid (EDTA), 1% sodium dodecylsulphate (SDS), 1% β-mercaptoethanol]. The homogenate was extracted twice with phenol–chloroform. RNA was precipitated with 4 M LiCl and resuspended in diethylpyrocarbonate-treated sterile water. To extract RNA from seedlings, 30 seedlings were ground in liquid nitrogen, and TriZol (Invitrogen, Carlsbad, CA, USA) was used according to the manufacturer's instructions. cDNA was generated using 1 μg of DNaseI (New England Biolabs, Ipswich, MA, USA)-treated RNA with an Oligo-dT primer and SuperscriptII (Invitrogen), following the manufacturer's instructions. RT-PCR was performed with the following cycling conditions: 95 °C for 30 s, 60 °C for 30 s and 68 °C for 40 s. The number of cycles varied from 26 to 32 depending on the gene. Real-time RT-PCRs were run using an Applied Biosystems (Carlsbad, CA, USA) model 7500 thermocycler with SYBR Green JumpStart Taq ReadyMix (Invitrogen). Results were analysed using a sigmoidal model for relative expression (Chervoneva *et al*., 2007; Rutledge and Stewart, 2008) and normalized to an internal standard, *PP2AA3* (At1g13320) (Czechowski *et al*., 2005). Primer pairs were designed to amplify all known splice variants of *JAZ* genes and are listed in Table S1. The *A. thaliana* genome sequence and annotation information used to design all the primers for this study were obtained from The Arabidopsis Information Resource TAIR (Swarbreck *et al*., 2008).

ACKNOWLEDGEMENTS

We thank Edward Farmer for providing the *JAZ10* RNAi line and for helpful comments on the manuscript. We are also grateful to Travis Dittmer, Andrzej Wierzbicki and Robert Kranz for critical discussions and technical advice, and to Andrew Mutka and Surobhi Lahiri for helpful discussions and comments. This study was supported by National Science Foundation grants IOB-0620469 and IOS-0818793.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Bacterial growth curves from experiments used for data collected in Figs 2 and 3. (a) Growth of *Pseudomonas syringae* strain DC3000 (diamond) and the DC3000 COR^- mutant DB29 (square) after dip infection of wild-type plants. Bacteria were isolated from leaves at the indicated times after inoculation. The zero time point represents bacterial levels approximately 1 h after inoculation. For the first time point, the leaves were surface sterilized with 15% H_2O_2 prior to grinding. Data points represent the average of four samples ± SE. (b) Growth of DC3000 after dip infection of wild-type (diamond) or *jin1-1* (triangle) plants. Data were collected and analysed as in (a).

Fig. S2. Additional independent experiment showing *JAZ* gene expression in *jin1* mutant plants during DC3000 infection. Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis of *JAZ* gene expression during DC3000 infection in wild-type (squares) and *jin1-1* (triangles) plants, and mock ($MgCl_2$) inoculation of wild-type (diamonds) plants. Tissue from uninfected plants was used for the 0-h time point. mRNA levels were monitored by real-time RT-PCR with *PP2AA3* (At1g13320) as an internal control. The expression of each gene at each time point is represented relative to *PP2AA3* (note different scales). Error bars represent ±SE of three technical replicates.

Fig. S3. Genotyping of *jaz10-1*. Agarose gel (2.5%) showing polymerase chain reaction (PCR) amplification products of genomic DNA from wild-type and *jaz10-1* plants using gene-specific primers (a) or a gene-specific primer and LB1, a T-DNA specific primer (b). Gene-specific primers that span the T-DNA insertion site amplify a slightly larger PCR product in *jaz10-1* plants than in wild-type plants.

Table S1 List of primers used for reverse transcription-polymerase chain reaction (RT-PCR), real-time RT-PCR and genotyping.

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