Regulation and Function of Arabidopsis *JASMONATE ZIM*-Domain Genes in Response to Wounding and Herbivory[1][W][OA]

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Jasmonate (JA) and its amino acid conjugate, jasmonoyl-isoleucine (JA-Ile), play important roles in regulating plant defense responses to insect herbivores. Recent studies indicate that JA-Ile promotes the degradation of JASMONATE ZIM-domain (JAZ) transcriptional repressors through the activity of the E_3 ubiquitin-ligase SCF^{COI1}. Here, we investigated the regulation and function of *JAZ* genes during the interaction of Arabidopsis (*Arabidopsis thaliana*) with the generalist herbivore *Spodoptera exigua*. Most members of the *JAZ* gene family were highly expressed in response to *S. exigua* feeding and mechanical wounding. *JAZ* transcript levels increased within 5 min of mechanical tissue damage, coincident with a large (approximately 25-fold) rise in JA and JA-Ile levels. Wound-induced expression of *JAZ* and other CORONATINE-INSENSITIVE1 (COI1)-dependent genes was not impaired in the *jar1-1* mutant that is partially deficient in the conversion of JA to JA-Ile. Experiments performed with the protein synthesis inhibitor cycloheximide provided evidence that *JAZ*s, *MYC2*, and genes encoding several JA biosynthetic enzymes are primary response genes whose expression is derepressed upon COI1-dependent turnover of a labile repressor protein(s). We also show that overexpression of a modified form of JAZ1 (JAZ1Δ3A) that is stable in the presence of JA compromises host resistance to feeding by *S. exigua* larvae. These findings establish a role for JAZ proteins in the regulation of plant anti-insect defense, and support the hypothesis that JA-Ile and perhaps other JA derivatives activate COI1-dependent wound responses in Arabidopsis. Our results also indicate that the timing of JA-induced transcription in response to wounding is more rapid than previously realized.

Jasmonate (JA) and its bioactive derivatives, collectively known as JAs, control many aspects of plant protection against biotic and abiotic stress. JAs play a central role in regulating immune responses to arthropod herbivores and necrotrophic pathogens, as well as stress responses to UV light and ozone (Devoto and Turner, 2005; Glazebrook, 2005; Gfeller et al., 2006; Wasternack et al., 2006; Wasternack, 2007; Balbi and Devoto, 2008; Browse and Howe, 2008; Howe and Jander, 2008). JAs also exert control over various developmental processes, including pollen maturation, anther dehiscence, embryo maturation, and trichome development (Li et al., 2004; Browse, 2005; Schaller et al., 2005). In general, JAs appear to promote defense and reproduction while inhibiting growth-related processes such as photosynthesis and cell division (Devoto and Turner, 2005; Giri et al., 2006; Yan et al., 2007). These contrasting activities of the hormone imply a broader role for the JAs in regulating tradeoffs between growth- and defense-oriented metabolism, thereby optimizing plant fitness in rapidly changing environments.

CORONATINE-INSENSITIVE1 (COI1) is an LRR (Leu-rich repeat)/F-box protein that determines the substrate specificity of the SCF-type E_3 ubiquitin ligase SCF^{COI1} (Xie et al., 1998; Xu et al., 2002). The importance of *COI1* in JA signaling is exemplified by the fact that null mutations at this locus abolish JA responses in diverse plant species (Feys et al., 1994; Li et al., 2004). JASMONATE ZIM-domain (JAZ) proteins are targeted by SCF^{COI1} for degradation during JA signaling (Chini et al., 2007; Thines et al., 2007). JAZ proteins belong to the larger family of proteins that share a conserved TIFY×G sequence within the ZIM motif (Vanholme et al., 2007). A second defining feature of JAZs is the highly conserved Jas motif, which has a $SLX_2FX_2KRX_2RX_5PY$ consensus sequence near the C terminus (Chini et al., 2007; Thines et al., 2007; Yan

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et al., 2007). Recent studies indicate that JAZ proteins act as repressors of JA-responsive genes. For example, JAZ proteins are degraded in a COI1- and 26S proteasome-dependent manner in response to JA treatment. Also, dominant mutations affecting the conserved Jas motif stabilize JAZ proteins against degradation and, as a consequence, reduce the plant's responsiveness to JA (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007). Current models indicate that, in the presence of low levels of JA, JAZ proteins repress the expression of JA-responsive genes by interacting directly with the bHLH (basic helix-loop-helix) transcription factor MYC2 (also known as JIN1), which is a positive regulator of JA responses (Lorenzo et al., 2004; Chini et al., 2007). Increased JA levels promote binding of JAZs to COI1 and subsequent degradation of JAZ repressors via the ubiquitin/26S proteasome pathway, resulting in derepression of primary response genes.

The JAZ-mediated transition between repressed and derepressed states of gene expression appears to be subject to several layers of regulation. It is well established, for example, that the expression of JA biosynthetic genes in Arabidopsis (*Arabidopsis thaliana*) and other plants increases in response to JA treatment and wounding (Reymond et al., 2000; Ryan, 2000; Sasaki et al., 2001; Ziegler et al., 2001; Stenzel et al., 2003; Delker et al., 2006; Ralph et al., 2006; Farmer, 2007; Wasternack, 2007). This observation implies the existence of a positive feedback loop that reinforces or amplifies the plant's capacity to synthesize JA in response to continuous tissue damage, such as that associated with biotic stress. Paradoxically, *JAZ* genes are also up-regulated in response to JA treatment. Because at least some JAZ proteins act as negative regulators, it was suggested that JA-induced *JAZ* expression constitutes a negative feedback loop in which newly synthesized JAZ repressors dampen the response by inhibiting the activity of MYC2 (Chini et al., 2007; Thines et al., 2007). This idea is analogous to the explanation for why auxin rapidly induces the expression of *Aux/IAA* genes, which encode negative regulators of the auxin signaling pathway (Abel et al., 1995; Abel, 2007). Indeed, the emerging picture of JA action is remarkably similar to that of the auxin signaling pathway in which auxin promotes the degradation of the Aux/IAA transcriptional repressors by the E_3 ubiquitin-ligase SCF^{TIR1} (Dharmasiri et al., 2005; Kepinski and Leyser, 2005; Tan et al., 2007).

Many plant antiherbivore defense responses are activated upon wound-induced accumulation of JA (Browse and Howe, 2008; Howe and Jander, 2008). The initial steps in the octadecanoid pathway for JA synthesis occur in the chloroplast, whereas the latter half of the pathway operates in the peroxisome (Schaller et al., 2005; Schilmiller and Howe, 2005; Wasternack et al., 2006; Wasternack, 2007). Analysis of mutants impaired in peroxisomal β-oxidation enzymes has shown that JA production is strictly required for defense against herbivorous caterpillars and thrips (Li et al., 2005; Schilmiller et al., 2007). It is now clear that metabolism of JA plays a critical role in regulating JA-based defenses. In particular, synthesis of the jasmonoyl-Ile (JA-Ile) conjugate by JASMONATE RESISTANT1 (JAR1; Staswick and Tiryaki, 2004) and related JA-conjugating enzymes is required for plant resistance to necrotrophic soil pathogens (Staswick et al., 1998), lepidopteran insects (Kang et al., 2006), and various abiotic stresses as well (Rao et al., 2000). Recent work by Thines et al. (2007) showed that JA-Ile, but not JA (i.e. jasmonic acid), methyl-JA (MeJA), or their chloroplastic C18 precursor 12-oxo-phytodienoic acid (OPDA), stimulates COI1 binding to JAZ proteins. Collectively, these results support the hypothesis that JA-Ile is a bioactive JA, which we define here as a compound that evokes a physiological response upon binding to a receptor.

In addition to regulation by exogenous JA (Chini et al., 2007; Thines et al., 2007), *JAZ* expression is also induced by high salinity and other environmental stress conditions (Jiang and Deyholos, 2006; Vanholme et al., 2007). Transcript profiling experiments in Arabidopsis (Yan et al., 2007) and hybrid poplar (Major and Constabel, 2006) showed that *JAZ* genes are up-regulated in response to wounding and simulated herbivory. Yan et al. (2007) also demonstrated that *JASMONATE-ASSOCIATED1* (*JAS1*), which is identical to *JAZ10*, is induced by mechanical wounding in a COI1-dependent manner. Moreover, a splice variant of *JAS1/JAZ10* that encodes a C-terminally truncated protein (JAS1.3) acts as a repressor of JA-mediated growth inhibition (Yan et al., 2007). This finding provides new mechanistic insight into JA's dual role in promoting defense and inhibiting growth. A role for JAZ proteins in mediating plant-herbivore interactions, however, remains to be established.

Here, we show that mechanical wounding and herbivory increase the expression of 11 of the 12 *JAZ* genes in Arabidopsis. We employed the protein synthesis inhibitor cycloheximide, JA measurements, and two well-defined JA mutants (*coi1-1* and *jar1-1*) to study the mechanism by which tissue damage activates the expression of *JAZ* and other primary response genes. Our results support a model in which wound-induced synthesis of one or more bioactive JAs triggers SCF^{COI1}-mediated degradation of JAZ repressors and subsequent expression of genes that further regulate the response both positively and negatively. These regulatory circuits have the potential to orchestrate host defenses that are commensurate with the intensity and duration of herbivore attack. We also provide evidence that JAZ proteins play a role in plant defense against insect herbivores.

RESULTS

Feeding by a Lepidopteran Herbivore Induces *JAZ* Expression

The central role of JA signaling in plant resistance to lepidopteran insects led us to investigate whether members of the Arabidopsis *JAZ* family are differen-

tially regulated in response to feeding by the generalist *Spodoptera exigua*. *S. exigua* larvae were allowed to feed on rosette leaves for either 2 or 24 h. Damaged (local) and undamaged (systemic) leaf tissue was harvested for RNA extraction and gel-blot analysis with gene-specific probes for each of the 12 members (*JAZ1*–*JAZ12*) in the Arabidopsis *JAZ* family (Chini et al., 2007; Thines et al., 2007; Vanholme et al., 2007). Insect feeding resulted in increased expression of all *JAZ*s, except *JAZ11*, in damaged leaves (Fig. 1A). Various members of the *JAZ* family were expressed at different levels in herbivore-challenged plants. For example, *JAZ1*, *JAZ2*, *JAZ5*, *JAZ6*, *JAZ9*, *JAZ10*, and *JAZ12* transcripts accumulated to relatively high levels in damaged leaves, whereas *JAZ3*, *JAZ4*, *JAZ7*, and *JAZ8* showed weaker expression. Herbivore-induced expression of *JAZ4* was very weak, and detection of these transcripts required prolonged exposure of autoradiographic films. In damaged leaves, transcript levels of most of the inducible *JAZ*s at the 24-h time point were similar to or greater than those at the 2-h time point. Several *JAZ*s (e.g. *JAZ1*) were also systemically expressed within 2 h of the onset of insect feeding, indicating that both the local and systemic response is relatively rapid (i.e. $<$2 h). These results demonstrate that feeding by a lepidopteran insect results in major reprogramming of *JAZ* expression, and that different *JAZ* genes exhibit distinct patterns of herbivore-induced expression.

The JA Pathway Mediates Rapid Induction of *JAZ* Genes in Response to Mechanical Wounding

We next performed RNA blot analyses to determine the *JAZ* expression pattern in rosette leaves subject to mechanical wounding with a hemostat. Similar to the results obtained with insect feeding, all *JAZ* mRNAs except *JAZ11* accumulated in mechanically damaged leaves (Fig. 1B). Expression of *JAZ1*, *JAZ2*, *JAZ5*, *JAZ6*, *JAZ7*, *JAZ8*, and *JAZ9* was strongly induced within 30 min of wounding, with mRNA levels declining at later time points. In contrast to these genes, wound-induced accumulation of *JAZ3*, *JAZ4*, *JAZ10*, and *JAZ12* mRNAs was delayed and weaker. Although the overall *JAZ* expression patterns elicited by mechanical wounding and herbivory by *S. exigua* were qualitatively similar, some quantitative differences were apparent. For example, we reproducibly observed that *JAZ7* and *JAZ8* mRNAs accumulated to lower levels (relative to other *JAZ* transcripts) in insect-damaged leaves compared to mechanically damaged leaves. Because the specific activity of radiolabeled *JAZ* probes and autoradiographic film exposure times were similar for each *JAZ* analyzed, this observation suggests that *JAZ7* and *JAZ8* expression is either enhanced by mechanical damage or suppressed by insect feeding.

Plants harboring null mutations in *COI1* provide a useful tool to determine the contribution of the JA pathway to the expression of wound-responsive genes (Feys et al., 1994; Devoto et al., 2005). To determine the extent to which COI1 regulates the wound-induced expression of *JAZ* genes, we assessed the expression pattern of selected *JAZ*s in wild-type and *coi1-1* plants (Fig. 2). *MYC2* expression was also analyzed in these experiments because this gene is known to be induced by wounding in a COI1-dependnt manner (Lorenzo et al., 2004). The results showed that accumulation of all wound-inducible *JAZ* mRNAs and *MYC2* was largely dependent on COI1 (Fig. 2). Prolonged exposure times of autoradiographic film (data not shown), however, indicated that all *JAZ*s were expressed at low levels in the *coi1* mutant. This experiment also showed that wound-induced accumulation of *MYC2* and several *JAZ* transcripts occurred within 15 min of leaf damage, which prompted us to further investigate the timing of the response.

Rapid Activation of *JAZ* Genes Is Correlated with JA and JA-Ile Accumulation

To define more precisely the timing of the wound response, we assessed the expression level of various genes at very early time points after wounding. The steady-state level of *JAZ1*, *JAZ5*, *JAZ7*, and *MYC2* transcripts increased within 5 min of wounding (Fig. 3A), as did the expression of *JAZ2*, *JAZ6*, and *JAZ9* (data not shown). Quantification of ^{32}P-labeled probe intensities on RNA blots showed that the level of *JAZ7* mRNA increased approximately 13-fold during the first 5 min after wounding. The strong dependence of wound-induced *JAZ* expression on COI1 (Fig. 2) indicated that increased expression of these genes is likely triggered by elevated levels of bioactive JAs. We used liquid chromatography-mass spectrometry to measure JA and JA-Ile levels at early time points after mechanical damage (Fig. 3, B and C). The levels of JA and JA-Ile in undamaged leaves were 29.5 $\pm$ 11.2 and 4.5 $\pm$ 1.3 pmol/g fresh weight (FW) tissue, respectively. These levels increased by approximately 25-fold (to 784 $\pm$ 99 and 111 $\pm$ 4, respectively) within the first 5 min after wounding. At the 30-min time point, JA and JA-Ile levels increased to 4,402 $\pm$ 499 and 972 $\pm$ 132 pmol/g FW, respectively. The steady increase in JA and JA-Ile levels during the first 30 min after wounding was tightly correlated with changes in gene expression.

Wound-Induced *JAZ* Expression Does Not Require JAR1

To test further the hypothesis that wound-induced, COI1-dependent expression of *JAZ* genes is mediated by JA-Ile, we analyzed the pattern of wound-induced gene expression in the *jar1-1* mutant that is impaired in the conversion of JA to JA-Ile (Staswick et al., 2002; Staswick and Tiryaki, 2004; Suza and Staswick, 2008). As shown in Figure 4, the level of *JAZ5*, *JAZ7*, and *MYC2* transcripts in wounded *jar1-1* plants was comparable to that in wild-type plants. Parallel analysis of the *coi1-1* mutant confirmed that the induced expression of these genes is dependent on an intact JA signaling pathway. Similar results were obtained for

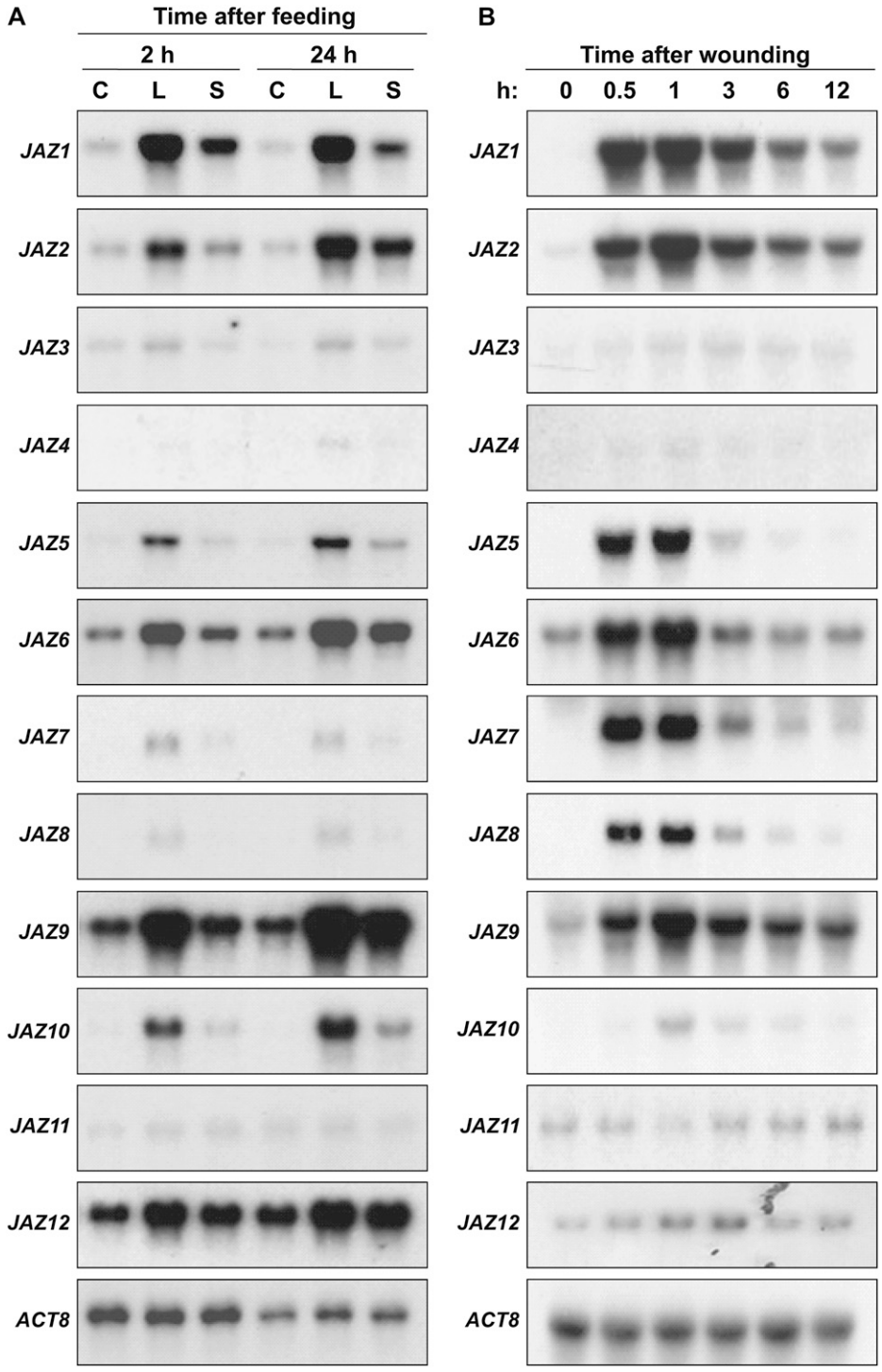

Figure 1. Expression of *JAZ* genes in response to herbivore feeding and mechanical wounding. A, Five-week-old wild-type plants were challenged with *S. exigua* larvae. At the indicated times (h) after feeding, damaged local (L) leaves and undamaged systemic (S) leaves were harvested for RNA extraction. A separate set of unchallenged plants was used as a control (C). Five micrograms of total RNA was loaded in each lane and blots were hybridized with the indicated cDNA probes. *ACTIN8* (*ACT8*) was used as a loading control. *JAZ4*- and *JAZ11*-probed blots were exposed to autoradiographic film for 16 h, whereas all other blots were exposed for 6 h. The contrast of *JAZ4*-probed blots was adjusted to facilitate visualization of the *JAZ4* signal. B, Five-week-old wild-type plants were wounded three times across the midrib with a hemostat and damaged leaves were collected for RNA extraction at the indicated times (h) after wounding. Ten micrograms of total RNA was loaded in each lane and blots were hybridized to gene-specific probes for each of the 12 *JAZ* genes, as well as *ACT8* as a loading control. *JAZ4*-, *JAZ11*-, and *ACT8*-probed blots were exposed to autoradiographic film for 16 h, whereas all other blots were exposed for 5 h.

two JA biosynthesis genes, *ALLENE OXIDE SYNTHASE* (*AOS*) and *12-OPDA REDUCTASE3* (*OPR3*), whose wound-induced expression is also COI1-dependent (Reymond et al., 2004; Devoto et al., 2005; Koo et al., 2006). These findings indicate that JAR1 activity is not strictly required for wound-induced expression of JA-responsive genes.

JAZ, *MYC2*, and JA Biosynthetic Genes Are Primary Response Genes in the JA Signaling Pathway

The current model of JA signaling indicates that *JAZ* genes are transcribed by MYC2 following degradation of one or more JAZ repressors in response to a bioactive JA signal (Chini et al., 2007; Thines et al., 2007).

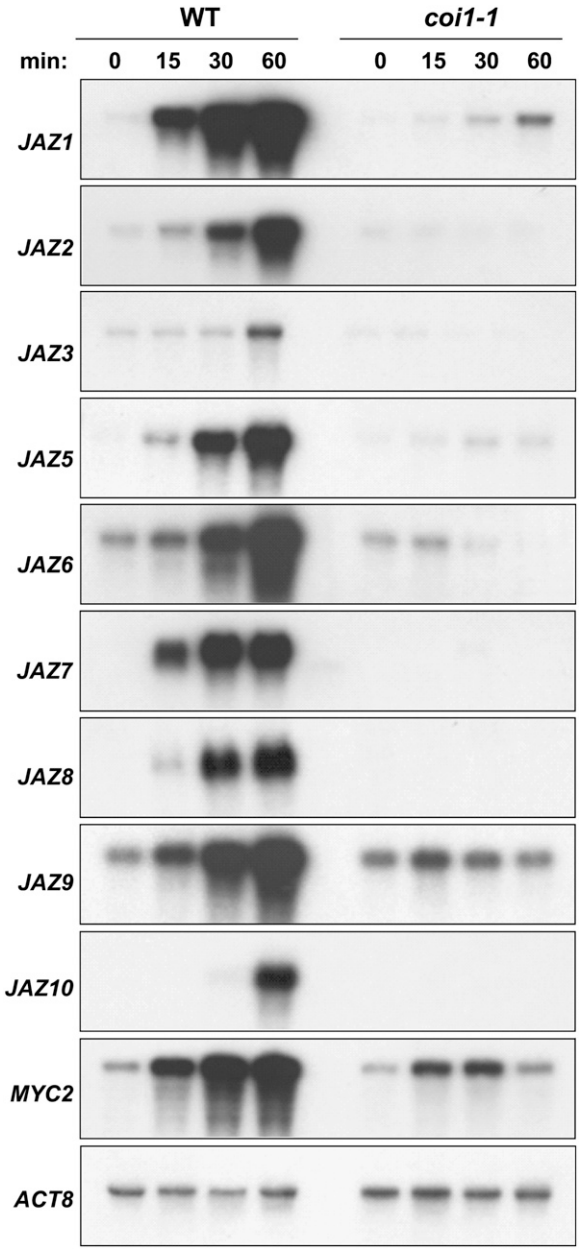

Figure 2. Effect of the *coi1-1* mutation on wound-induced expression of *JAZs*. Mechanical wound treatments and RNA gel-blot analysis were performed as described in the legend to Figure 1B. Damaged leaves were collected for RNA extraction at the indicated times (min) after wounding. Blots were hybridized to gene-specific probes for each of the indicated *JAZ* genes, *MYC2*, and *ACT8* as a loading control. All blots were exposed to autoradiographic film for 8 h.

This model implies that *JAZ*s are primary response genes in the JA signaling pathway, which is consistent with their rapid induction following mechanical wounding (Fig. 3A). To test directly whether *JAZ*s are primary response genes, we used the protein synthesis inhibitor cycloheximide (CHX) to determine whether JA-induced expression of *JAZ*s and *MYC2* requires de novo protein synthesis. Treatment of liquid-grown seedlings with MeJA induced the expression of *JAZ*s and *MYC2*, as expected (Fig. 5A). CHX treatment resulted in the accumulation of *MYC2*, *JAZ1*, *JAZ10*, and all other *JAZ* transcripts except *JAZ11* (Fig. 5A; data not shown). Induction of *JAZ*s and *MYC2* by MeJA was not inhibited by CHX. Rather, seedlings treated with both MeJA and CHX accumulated higher levels of these mRNAs than seedlings treated with either compound alone (Fig. 5A). These results indicate that *JAZ*s and *MYC2* are primary response genes (i.e. they are transcribed in the absence of de novo protein synthesis). *VSP1* and *LOX2* were used as markers for secondary response genes. In agreement with previous reports (Rojo et al., 1998; Jensen et al., 2002), we found that MeJA-induced expression of *VSP1* and *LOX2* was blocked by CHX. The conclusion that *JAZ*/*MYC2* and *VSP1*/*LOX2* are primary and secondary response genes, respectively, is supported by differences in their temporal expression patterns: *JAZ* and *MYC2* transcript levels peaked early (e.g. 0.5 h) after MeJA treatment, whereas *VSP1* and *LOX2* expression was delayed and more gradual.

We used the Expression Angler data-mining tool (Toufighi et al., 2005) to identify genes that are coregulated with *JAZ*s. Among the genes that were consistently identified as being coexpressed with *JAZ*s and *MYC2* in both hormone and pathogen data sets were several JA biosynthetic genes, including *AOS*, *OPR3*, *LIPOXYGENASE3* (*LOX3*), *LOX4*, *ALLENE OXIDE CYCLASE3* (*AOC3*), and *OPC-8:0 CoA LIGASE1* (*OPCL1*; Supplemental Table S1). *LOX2* was not identified in this list of coregulated genes. We therefore hypothesized that, like *JAZ* and *MYC2*, coregulated JA biosynthesis genes are primary response genes. To test this idea, we compared the effects of MeJA and CHX treatments on the expression of JA biosynthesis genes to those of *JAZ* and *MYC2*. As shown in Figure 5A, the MeJA- and CHX-induced expression patterns of *LOX3*, *LOX4*, *AOS*, *AOC3*, *OPR3*, and *OPCL1* were very similar to those of *JAZ1*, *JAZ10*, and *MYC2*. Specifically, the MeJA-induced expression of these JA biosynthesis genes was not inhibited by CHX, and the effects of MeJA and CHX were additive. Moreover, the timing of MeJA-induced expression of these JA biosynthesis genes, with the exception of *AOC3*, was similar to that of *MYC2* and *JAZ1*/*JAZ10*.

The JAZ repressor model predicts that CHX-induced expression of primary response genes results from cellular depletion of one or more JAZ repressors. Because CHX blocks de novo synthesis of JAZ proteins, the ability of CHX alone to activate primary response genes (Fig. 5A) suggests that JAZ repressors are highly unstable in wild-type seedlings, even in the absence of exogenous JA. To test the hypothesis that SCF^{COI1} contributes to JAZ turnover in the absence of exogenous MeJA, we determined the expression pattern of *JAZ*, *MYC2*, and JA biosynthesis genes in wild-type and *coi1* seedlings treated with either CHX or a mock control (Fig. 5B). CHX-induced accumulation of primary gene transcripts was severely attenuated in

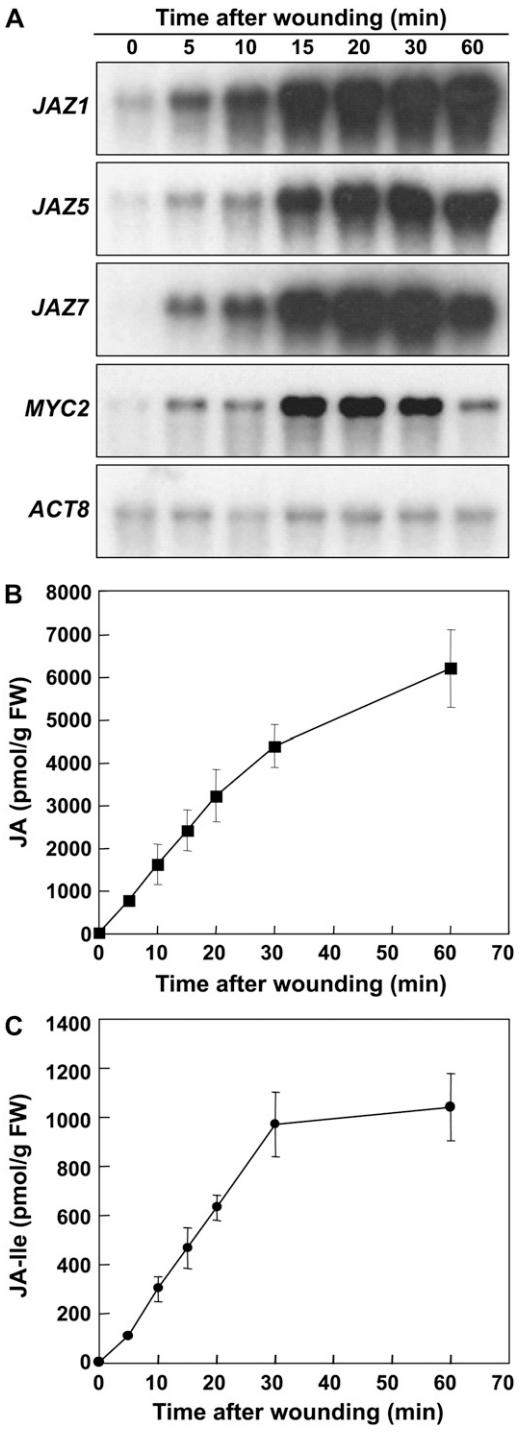

Figure 3. Rapid induction of *JAZ* transcripts and accumulation of JAs in response to mechanical wounding. A, RNA gel-blot analysis of *JAZ* expression in wounded leaves. Wound treatments and northern-blot analyses were performed as described in the Figure 1B legend. Damaged leaves were collected for RNA extraction at the indicated times (min) after wounding. *MYC2*- and *JAZ*-probed blots were exposed to autoradiographic film for 4 and 14 h, respectively. B and C, Time course of JA (B) and JA-Ile (C) accumulation in response to mechanical wounding. Leaf tissue from the same set of plants used for RNA blot analysis (A) was harvested at the indicated time points after wounding for extraction of JAs, as described in "Materials and Methods". JA and JA-Ile (measured as the total of JA-Ile plus JA-Leu) levels were determined by liquid chromatography-mass spectrometry according to the procedure described in "Materials and Methods." Each data point represents the mean ±SD of four biological replicates.

coi1 compared to wild-type seedlings. Interestingly, the accumulated level of *JAZ1* and *MYC2* mRNAs in CHX-treated *coi1* plants was much greater than that of other genes tested (*JAZ7*, *JAZ10*, *AOS*, and *OPR3*). CHX-induced expression of *JAZ2*, *JAZ5*, and *JAZ9* was also strongly suppressed in *coi1* plants (data not shown). These results are consistent with the idea that COI1 promotes the turnover of JAZ repressors even in the absence of exogenous JA.

Wound-Induced JA Accumulation Is Dependent on COI1

The finding that CHX-induced expression of *AOS* and *OPR3* is dependent on COI1 (Fig. 5B) is consistent with other studies showing that wound- and JA-induced expression of these genes requires COI1 (Titarenko et al., 1997; Reymond et al., 2000; Cruz Castillo et al., 2004; Devoto et al., 2005; Koo et al., 2006). To determine the role of COI1 in wound-induced JA accumulation, we used gas chromatography-mass spectrometry to measure JA levels in unwounded (control) and mechanically damaged leaves of wild-type and *coi1* plants (Fig. 6). The basal level of JA in unwounded wild-type and *coi1* plants was not significantly different (0.20 ± 0.07 and 0.19 ± 0.09 nmol/g FW tissue, respectively). The JA content in wild-type plants increased rapidly after wounding, with peak levels (6.94 ± 0.42 nmol JA/g FW) attained 1 h after treatment. In comparison to this robust response, wounded *coi1* leaves were severely deficient in JA accumulation. Mechanical wounding increased the JA content in wild-type and *coi1* leaves by approximately 35-fold and 4-fold, respectively, at the 1-h time point. The amount of JA in *coi1* leaves at all time points after wounding ranged between 9% and 14% of wild-type levels. These results demonstrate that COI1 activity plays an important role in promoting the accumulation of JA in wounded Arabidopsis leaves.

Disruption of JA Signaling by a Truncated Form of JAZ1 Compromises Resistance to *S. exigua* Feeding

JAZ proteins that lack the C-terminal Jas motif reduce the plant's sensitivity to JA and, as a consequence, cause several JA-related phenotypes (Chini et al., 2007, Thines et al., 2007, Yan et al., 2007). To test whether such truncated JAZ derivatives alter host resistance to herbivory, we compared the defense response of *S. exigua*-challenged wild-type plants to that of a transgenic line (Thines et al., 2007) expressing a Jas-motif-deleted form (JAZ1Δ3A) of JAZ1. As shown in Figure 7, A and B, larvae reared on *JAZ1Δ3A* plants gained significantly more weight than larvae grown on wild-type plants (Student's *t* test, $P < 0.0001$). Thus,

perturbation of JA signaling by overexpression of JAZ1Δ3A decreases host resistance to *S. exigua* feeding. RNA blot analysis was used to determine the effect of JAZ1Δ3A on the expression of various wound-response genes in *S. exigua*-challenged plants. In wild-type plants subjected to insect feeding for 13 d, *MYC2*, *JAZ1*, *JAZ5*, *OPR3*, and *VSP1* transcripts were highly elevated in comparison to untreated control plants (Fig. 7C). Herbivore-induced levels of *MYC2*, *JAZ1*, *JAZ5*, and *OPR3* mRNAs in *JAZ1Δ3A* plants were significantly less than those in the wild type. The expression level of *VSP1* in insect-damaged *JAZ1Δ3A* plants, however, was similar to that in wild-type plants (Fig. 7C). These findings indicate that decreased resistance of *JAZ1Δ3A* plants to *S. exigua* feeding is correlated with reduced expression of some, but not all, JA responsive genes.

DISCUSSION

Wound-Induced Expression of *JAZ* Genes in Arabidopsis

The recent discovery of JAZ proteins as negative regulators of JA signaling marks an important advance in our mechanistic understanding of how plants respond to biotic stress through changes in growth- and defense-related processes (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007). Given the central role of JAs in the control of induced resistance to insect attack, we initiated this study with the goal of determining how the *JAZ* gene family in Arabidopsis is regulated in response to mechanical wounding and herbivory. With the exception of *JAZ11*, levels of all *JAZ* transcripts

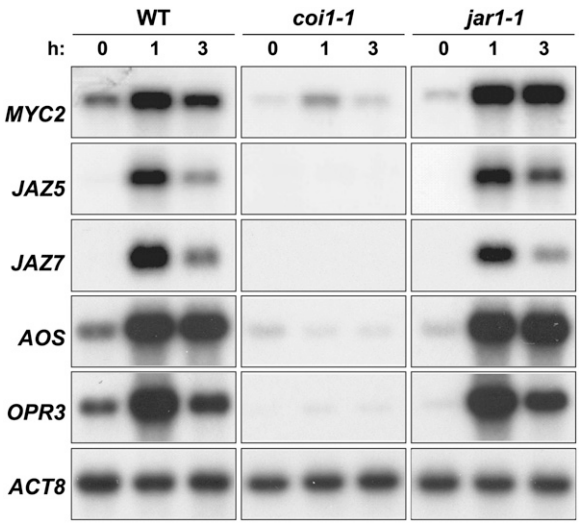

Figure 4. Wound-induced expression of JA-responsive genes in the *jar1-1* mutant. Five-week-old wild-type, *coi1-1*, and *jar1-1* plants were mechanically wounded as described in the legend to Figure 1B. Damaged leaves were collected for RNA extraction at the indicated times (h) after mechanical wounding. Blots were hybridized to probes for *MYC2, JAZ5, JAZ7,* two JA biosynthesis genes (*AOS* and *OPR3*), as well as *ACT8* as a loading control. All blots except *ACT8* were exposed to autoradiographic film for 6 h. The *ACT8* blot was exposed for 16 h.

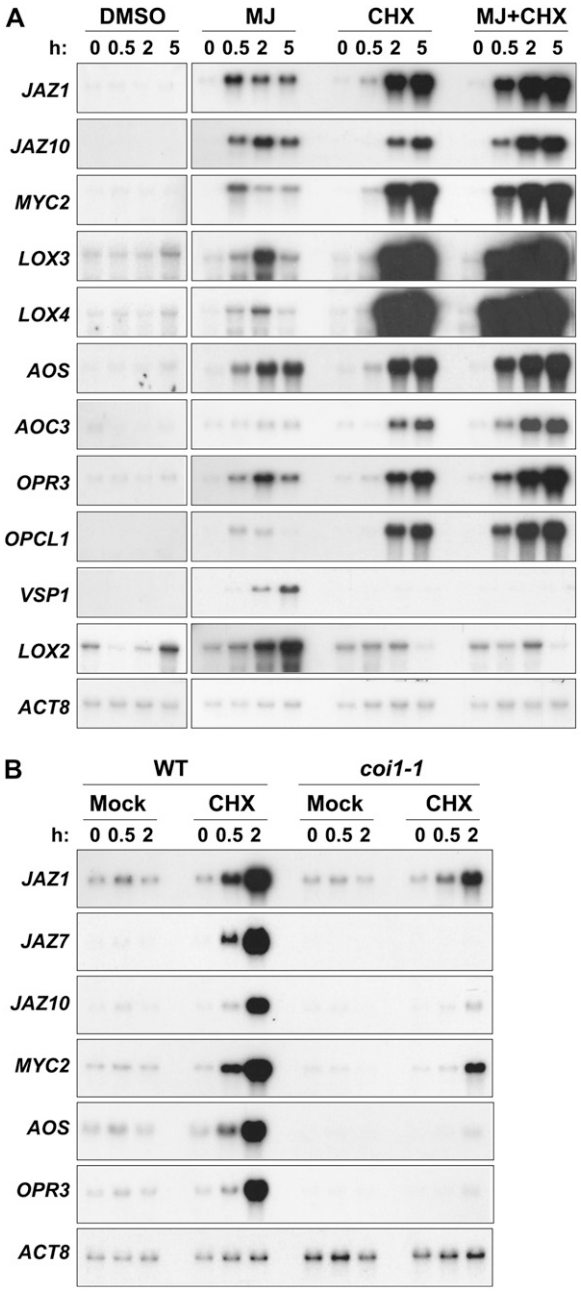

Figure 5. Effect of cycloheximide treatment on JA-responsive genes. A, Twelve-day-old wild-type seedlings grown in liquid medium were treated with either a mock control (0.2% DMSO), 50 μM MeJA (MJ), 50 μM cycloheximide (CHX), or a combination of MeJA and CHX (MJ + CHX) as described in "Materials and Methods". Whole seedlings were collected for RNA extraction at the indicated times (h) after treatment. Five micrograms of total RNA was loaded in each lane and blots were hybridized to the indicated probes (see text). *ACT8* was used as a loading control. *LOX3*- and *LOX4*-probed blots were exposed to film for 14 h, whereas all other blots were autoradiographed for 3 h. B, Effect of *coi1-1* on CHX-induced expression of JA-responsive genes. CHX treatment and RNA gel-blot analysis were performed as described above. All blots were autoradiographed for 4 h.

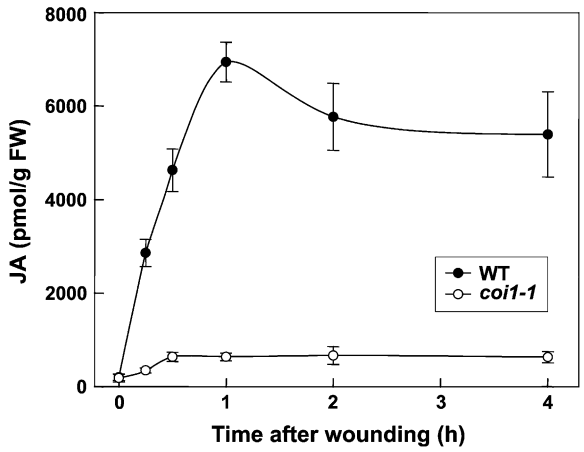

Figure 6. *coi1-1* plants are deficient in wound-induced accumulation of JA. Rosette leaves on 5-week-old wild-type (black circles) and *coi1-1* mutant (white circles) plants were mechanically wounded at the distal end with a hemostat. Wounded leaves were harvested for JA extraction at the indicated times (h) after wounding. Unwounded leaf tissue was used as a control for the 0-h time point. Each data point represents the mean ±SD of three samples from independent sets of plants.

increased in response to both mechanical wounding and feeding by *S. exigua* larvae. The various wound-responsive *JAZ*s showed differences in the timing and amplitude of expression. Most *JAZ*s (e.g. *JAZ1*) were expressed strongly and rapidly (i.e. <0.5 h) in response to mechanical wounding. Induced expression of other *JAZ*s, including *JAZ3*, *JAZ4*, *JAZ10*, and *JAZ12*, was temporally delayed and relatively weak by comparison. Our results are in good agreement with previous studies showing that most *JAZ* genes are rapidly induced by JA treatment (Chini et al., 2007; Thines et al., 2007), and that some Arabidopsis *JAZ*s are wound responsive (Yan et al., 2007). Wound-induced expression of *JAZ* genes in poplar (*Populus* spp.; Major and Constabel, 2006) and tomato (*Solanum lycopersicum*; L. Katsir and G.A. Howe, unpublished data) has also been observed, indicating that this phenomenon is conserved in the plant kingdom.

All *JAZ* genes induced by mechanical wounding were also induced by *S. exigua* feeding. This finding is consistent with studies showing that mechanical tissue damage and herbivory (or simulated herbivory) elicit similar, although not identical, changes in gene expression (Reymond et al., 2000; Mithofer et al., 2005; Major and Constabel, 2006; Ralph et al., 2006). We cannot exclude the possibility that mechanical wounding and herbivory elicit quantitative differences in *JAZ* expression. It is interesting to note, for example, that *JAZ7* and *JAZ8* mRNAs accumulated to lower levels in insect-damaged leaves compared to mechanically damaged leaves, which suggests that *JAZ7* and *JAZ8* expression may be suppressed by insect feeding. Previous studies have provided evidence for compounds in insect oral secretions that suppress the expression of host plant defenses (Schittko et al., 2001; Musser et al., 2005).

The physiological significance of wound-induced *JAZ* expression remains to be determined. Based on the function of JAZ proteins as repressors of JA-responsive genes, however, it was suggested that rapid synthesis of new JAZ proteins during JA signaling

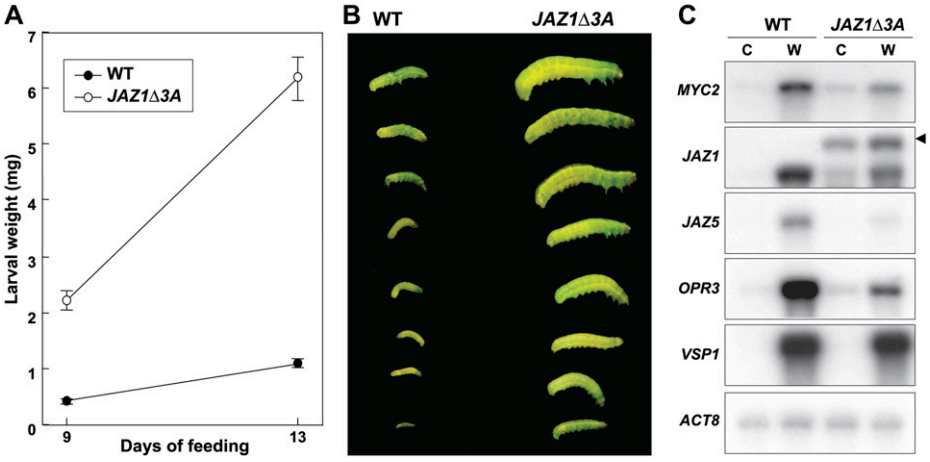

Figure 7. *JAZ1Δ3A* plants are compromised in resistance to feeding by *S. exigua*. A, Newly hatched *S. exigua* larvae were reared on wild-type (WT in the image) and *JAZ1Δ3A* transgenic plants. Larval weights were measured 9 and 13 d after the start of the feeding trial. Values indicate the mean ±SE. The number of wild-type-reared larvae at the 9- and 13-d time points was 79 and 73, respectively, whereas the number of *JAZ1Δ3A*-reared larvae was 87 and 111, respectively. B, Representative *S. exigua* larvae recovered wild-type and *JAZ1Δ3A* plants at the 13-d time point. C, Expression of various wound-responsive genes in undamaged control (C) and *S. exigua*-damaged (W) wild-type and *JAZ1Δ3A* plants. The arrow in C denotes a higher-M_r *JAZ1* transcript that presumably is derived from the *JAZ1Δ3A-GUS* transgene. RNA was extracted from *S. exigua*-damaged leaves collected at the 13-d time point, or from a set of undamaged plants grown in parallel. Northern-blot analyses were performed as described in the Figure 1 legend.

serves to attenuate the transcriptional response soon after it is initiated (Thines et al., 2007). In the context of plant defense responses to herbivory, wound-induced production of JAZ proteins may provide a mechanism to restrain the expression of energetically demanding defensive processes. Such restraint may be particularly important when JA levels decline, for example, upon cessation of insect feeding. This putative mechanism of negative feedback control suggests that JA-mediated defenses operate more as a dynamic continuum than as discrete induced and uninduced states.

Rapid Wound-Induced Expression of *JAZ*s Is Mediated by the JA Pathway

The dependence of wound-induced *JAZ* expression on COI1 (Fig. 2; Yan et al., 2007) indicates that a bioactive JA signal(s) produced in wounded leaves triggers SCF^{COI1}/26S proteasome-mediated destruction of JAZ repressors and subsequent transcription of primary response genes. The correlation between gene expression and accumulation of JA and JA-Ile in damaged leaves (Fig. 3) suggests that JA and/or JA-Ile could function as the active wound signal. The ability of JA-Ile, but not JA/MeJA, to promote COI1 interaction with JAZ1 argues in favor of JA-Ile as this signal, as does the established role of this conjugate in plant responses to biotic stress (Staswick et al., 1998; Kang et al., 2006). Surprisingly, however, wound-induced expression of COI1-dependent genes in the JA-Ile-deficient *jar1-1* mutant was not significantly impaired (Fig. 4), indicating that JAR1 is not strictly required for the response. This conclusion is in agreement with a recent study by Suza and Staswick (2008). One interpretation of this finding is that JA is nonbioactive (i.e. not a receptor ligand) and that the *jar1-1* mutant produces a sufficient amount of JA-Ile to promote COI1-JAZ interactions that de-repress the expression of wound responsive genes. Indeed, Suza and Staswick (2008) reported that JA-Ile levels in wounded *jar1-1* leaves are approximately 10% of wild-type levels. In response to the severe mechanical wound treatment used in our experiments, we observed that leaves of a *jar1* null mutant accumulate ~25% of the wild-type level of JA-Ile (A.J.K. Koo and G.A. Howe, unpublished data). The pool of JA-Ile in *jar1-1* plants results from the activity of at least one other JA-conjugating enzyme (Suza and Staswick, 2008). Identification of this enzyme should facilitate the important goal of generating Arabidopsis mutants with more severe JA-Ile-deficient phenotypes. An alternative explanation for our results is that JA or a JA derivative whose synthesis does not depend on JAR1 is a bioactive signal for COI1-dependent gene expression. This idea is supported by recent work indicating that JA complements the function of JA-Ile in promoting defense responses in *Nicotiana attenuata* (Wang et al., 2008). The hypothesis that JA is bioactive per se predicts the existence of JAZ proteins whose interaction with COI1 is promoted by JA. It will be interesting to determine the molecular specificity of the complete repertoire of JAZ proteins in plants such as Arabidopsis that have a well-defined JAZ family.

Positive Feedback Regulation of JA Biosynthesis Is a Primary Response of JA Signaling

Hormone-induced changes in physiology typically involve the expression of primary response genes that, in turn, control secondary transcriptional responses. The protein synthesis inhibitor CHX provides a useful tool to identify primary and secondary response genes in the JA signaling pathway (van der Fits and Memelink, 2001; Pauw and Memelink, 2005). The ability of CHX to block MeJA-induced expression of *LOX2* and *VSP1* indicates that these genes are secondary response genes, in agreement with previous studies (Rojo et al., 1998; Jensen et al., 2002). In contrast to *LOX2* and *VSP1*, the insensitivity of MeJA-induced *MYC2* and *JAZ* expression to CHX indicates that these genes can be classified as primary response genes. This interpretation is consistent with the ability of MYC2 to recognize the G-box motif found in the promoter of *JAZ* genes, and the proposed direct inhibitory action of JAZ3 on MYC2 (Chini et al., 2007). There is also evidence to indicate that MYC2 binds to a G-box motif in the *MYC2* promoter, thereby regulating its own transcription (Dombrecht et al., 2007). We suggest that CHX-induced turnover of JAZ repressors releases JAZ-mediated inhibition on MYC2, which is then free to transcribe *JAZ*, *MYC2*, and other target genes. Our results differ from those of Dombrecht et al. (2007), who reported that *MYC2* is a secondary response gene. These workers also reported that the expression of *VSP1*, although a secondary response gene, is induced by CHX, whereas our results (Fig. 5A) and those of Rojo et al. (1998) indicate that *VSP1* is not induced by CHX. These discrepancies may reflect differences in methods used for CHX treatment and transcript quantification.

Several studies have shown that Arabidopsis genes encoding JA biosynthetic enzymes are up-regulated via the JA/COI1 pathway in response to wounding and JA treatment (Reymond et al., 2000; Sasaki et al., 2001; Stenzel et al., 2003; Devoto and Turner, 2005; Koo et al., 2006). The generally accepted view of this regulatory phenomenon is that it provides a positive feedback mechanism to reinforce or amplify the plant's capacity to synthesize JA in response to long-term environmental (e.g. herbivory) or developmental cues (Stenzel et al., 2003; Farmer, 2007; Wasternack, 2007). Although the sensitivity of MeJA-induced *LOX2* expression to CHX suggests that this feedback mechanism is a secondary response, our results indicate that many other known or putative JA biosynthetic genes are primary targets of JA signaling. First, we observed that *AOS*, *AOC3*, *OPR3*, *OPCL1*, *LOX3*, and *LOX4* (but not *LOX2*) are tightly coregulated with *MYC2* and *JAZ*s (Supplemental Table S1). Second, these biosynthetic genes were induced by CHX treatment, and superinduced in response to treatment with both MeJA

and CHX. Finally, CHX-induced expression of *AOS* and *OPR3* was largely dependent on COI1. We thus conclude that JA biosynthetic genes, like *JAZ* genes, are negatively regulated by one or more labile proteins whose turnover is dependent on COI1 activity. JAZ proteins are obvious candidates for such repressors.

Among the five LOXs in Arabidopsis, LOX2 is the only isoform known to be involved in JA biosynthesis (Bell et al., 1995). The sequences of LOX3 and LOX4 predict that they are 13-LOXs that, like LOX2, catalyze formation of JA precursors in the plastid (Feussner and Wasternack, 2002; Liavonchanka and Feussner, 2006). The coexpression of *LOX3* and *LOX4* with other JA biosynthesis genes (Fig. 5; Supplemental Table S1) leads us to speculate that these LOXs may also serve a role in JA synthesis. Rigorous testing of this idea will require analysis of *lox3* and *lox4* mutants.

We found that *coi1* plants are severely deficient in wound-induced JA accumulation. By demonstrating that JA accumulation per se is decreased in a JA signaling mutant, this observation extends previous studies (e.g. Stenzel et al., 2003) showing that the expression of JA biosynthestic genes and enzymes is regulated by a positive feedback loop (Farmer, 2007; Wasternack, 2007). Moreover, our identification of JA biosynthesis genes as primary response genes implies that this positive feedback mechanism is engaged very rapidly after wounding. Given the well-documented JA/COI1-dependent expression of JA biosynthesis genes, a likely interpretation of our results is that *coi1* leaves contain limited amounts of one or more JA biosynthetic enzymes. Support for this idea comes from the observation that unwounded leaves of the JA-deficient *opr3* mutant contain significantly reduced levels of AOC protein (Stenzel et al., 2003). This scenario for the *coi1* mutant is clearly different from wild-type plants in which wound-induced JA biosynthesis is limited by substrate availability rather than by the level of octadecanoid pathway enzymes (Stenzel et al., 2003; Wasternack, 2007). It is also possible that the JA deficiency in wounded *coi1* leaves reflects reduced amounts of the initial substrate for JA synthesis, or the increased activity in the mutant of an enzyme that metabolizes JA. The former hypothesis is supported by recent work showing that *coi1* plants are deficient in the accumulation of OPDA- and dinor-OPDA-containing galactolipids that may function as precursors for JA synthesis (Buseman et al., 2006; Kourtchenko et al., 2007).

Regulation of Primary Response Genes by JAZ Repressors

The identification of JAZ proteins as negative regulators that link the action of SCF^{COI1} to transcription factors such as MYC2 has led to a relatively simple model of JA signaling (Chini et al., 2007; Thines et al., 2007). One prediction of this model is that the JA-insensitive phenotype of *coi1* plants results from the accumulation of JAZ repressors. Our results provide indirect support of this idea. First, wound-responsive *JAZ* genes exhibit low basal expression in the *coi1* mutant, indicating that JAZ proteins are likely synthesized in the *coi1* mutant. Similar results were obtained for *JAZ* genes in the COI1-deficient *jai1-1* mutant of tomato (L. Katsir and G.A. Howe, unpublished data). Second, our data showing that CHX-induced accumulation of *JAZ* transcripts is attenuated in *coi1* seedlings is consistent with the idea that JAZ proteins are destabilized by SCF^{COI1}-mediated ubiquitination (Chini et al., 2007; Thines et al., 2007). Taken together, these findings imply that JAZ proteins are more stable in the absence of SCF^{COI1} ligase activity and, as a consequence, accumulate in *coi1* plants to levels that effectively repress gene expression. This model predicts that JAZ repressors also accumulate in mutants that are deficient in JA synthesis. Measurement of JAZ protein levels in wild-type, *coi1*, and JA synthesis mutants will provide an important test of this hypothesis.

It is interesting to note that the *coi1* mutation had a differential effect on CHX-induced expression of various primary response genes. For example, *coi1* nearly abolished CHX-induced accumulation of *JAZ7* mRNA, whereas *JAZ1* and *MYC2* transcripts persisted to higher levels in CHX-treated *coi1* seedlings. One interpretation of this finding is that different *JAZ* genes are repressed by different JAZ proteins. For example, rapid accumulation of *JAZ7* transcripts in CHX-treated wild-type, but not *coi1*, seedlings suggests that the JAZ repressor of *JAZ7* is relatively stable in the absence of COI1. Likewise, the putative JAZ repressor of *JAZ1* and *MYC2* would appear to be less stable in the absence of COI1. Chini et al. (2007) demonstrated that MYC2 interacts directly with JAZ3. Because MYC2 is implicated in the transcriptional regulation of most *JAZ* genes (Chini et al., 2007), we speculate that JAZ proteins other than JAZ3 also inhibit MYC2 function. Other interpretations, including differential distribution of positively acting transcription factors at various *JAZ* promoters, or differences in the stability of *JAZ* mRNAs, may also explain why *coi1* differentially affects CHX-induced expression of different primary response genes.

A Role for JAZ Proteins in Defense against Insect Herbivores

A direct role for *JAZ* genes in plant-herbivore interactions has not been previously reported. Our finding that *S. exigua* larvae reared on *JAZ1Δ3A* plants gained significantly more weight than larvae reared on wild-type plants (Fig. 7) provides evidence that JAZ proteins do indeed play an important role in regulating plant processes that confer resistance to insect herbivores. The increased susceptibility of *JAZ1Δ3A* plants to *S. exigua* can most likely be attributed to the fact that this mutant exhibits decreased responsiveness to JA and several other *coi1*-like phenotypes, including male sterility (Thines et al., 2007). The reduced accumulation of some JA/wound-responsive transcripts in herbivore-challenged *JAZ1Δ3A* plants is consistent

with this interpretation. Moreover, recent studies have shown that *S. exigua* larvae perform better on *coi1* than wild-type plants (Mewis et al., 2005, 2006).

JAZ1Δ3A mutants are presumably deficient in defensive compounds that normally act to deter *S. exigua* feeding on wild-type plants. Some Arabidopsis VSPs are expressed in a COI1-dependent manner and are known to function as anti-insect proteins (Benedetti et al., 1995; Liu et al., 2005). However, because herbivore-treated *JAZ1Δ3A* plants were not significantly affected in *VSP1* expression, it seems unlikely that a deficiency in these proteins can explain the increased susceptibility of the transgenic line. Mewis and coworkers demonstrated that increased performance of *S. exigua* on the *coi1* mutant correlates with reduced production of glucosinolates, which have a well-established role in defense against generalist herbivores such as *S. exigua* (Mewis et al., 2005, 2006). This observation raises the possibility that *JAZ1Δ3A* plants are defective in glucosinolate-based defenses. Transgenic expression of JAZ1Δ3A or other C-terminally truncated JAZs may provide a useful approach to elucidate specific COI1-dependent processes that confer plant protection to insect herbivores and other forms of environmental stress.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia-0 (Col-0) was used as the wild type for all experiments. Soil-grown plants were maintained in a growth chamber at 21°C under 16-h light (100 μE m^{-2} s^{-1}) and 8-h dark. For growth of seedlings in liquid media, seeds were surface-sterilized with 30% (v/v) commercial bleach for 15 min and washed 10 times with sterile water. Approximately 100 seeds were placed in 50 mL of Murashige and Skoog (MS) medium in a 125-mL Erlenmeyer flask. The flasks were placed at 4°C for 4 d in darkness, and then incubated under normal growth conditions (described above) for 12 d prior to treatment. Flasks were rotated on an orbital shaker (150 rpm) for the duration of the experiment. Seeds collected from heterozygous *coi1-1* plants (Feys et al., 1994) were germinated on MS medium containing 50 μM MeJA to select for JA-insensitive *coi1-1* homozygous plants, which were then transferred either to soil or MS liquid medium for further experiments. Seed for the *jar1-1* mutant (Staswick et al., 2002) was obtained from the Arabidopsis Biological Resource Center. The JA-insensitive root growth phenotype of *jar1-1* plants was verified by germinating seeds on MeJA-containing MS medium (Staswick et al., 2002). A male sterile line of Arabidopsis expressing the *35S-JAZ1Δ3A-GUS* transgene (Thines et al., 2007) was propagated by outcrossing to wild-type pollen. F_1 progeny containing the transgene were selected on MS medium containing kanamycin (50 μg/mL).

Plant Treatments

Spodoptera exigua eggs were obtained from Benzon Research and hatched at 27°C. For the insect feeding experiment shown in Figure 1A, newly hatched larvae were transferred to a petri dish and reared on Arabidopsis leaves for 3 to 4 d. Prior to the feeding experiment, second instar larvae were transferred into a new petri dish and starved for 14 h. Approximately 10 larvae were transferred to fully expanded rosette leaves (two to three larvae per leaf) on 5-week-old plants. Insect-challenged and control unchallenged plants were maintained under continuous light at 26°C. Two hours after transfer of larvae to the plants, insect-damaged leaf tissue was harvested for RNA extraction. Approximately 5% of the leaf area (local response) was removed by feeding at this time point. A second set of plants was used to collect tissue for the 24-h time point, at which time 20% to 60% of the leaf area was damaged by herbivory. Undamaged leaves from challenged plants were harvested at both the 2- and 24-h time points to determine the effect of insect feeding on systemic expression of *JAZ* genes.

For the herbivore performance assay shown in Figure 7, newly hatched *S. exigua* larvae were transferred to 5-week-old wild-type and *JAZ1Δ3A-GUS* plants. Eight larvae were reared on each of 48 wild-type and 48 transgenic plants. Plants were maintained under standard growth conditions (see above). The weight of individual larvae was determined 9 d after the start of the feeding trial. Larvae were returned to the same set of plants and, after 4 additional days of feeding, were weighed again.

For mechanical wound treatments, fully expanded rosette leaves on 5-week-old plants were wounded three times by crushing the leaf across the midrib with a hemostat. This wounding protocol, which resulted in damage to approximately 40% of the leaf area, was administered to approximately six rosette leaves per plant. At various times after wounding, damaged leaves were harvested, immediately frozen in liquid nitrogen, and stored at −80°C until use for RNA and extraction of JAs.

Stock solutions (100 mM) of MeJA and CHX (Sigma) in dimethyl sulfoxide (DMSO) were added to liquid cultures of Arabidopsis seedlings (see above) to a final concentration of 50 μM. To determine the effect of CHX treatment on MeJA-induced gene expression, liquid-grown seedlings were pretreated with 50 μM CHX for 1.5 h prior to the addition of MeJA. Seedlings were treated with 0.2% (v/v) DMSO as a mock control. At various times after treatment, seedlings were harvested, frozen in liquid nitrogen, and stored at −80°C until needed for RNA extraction.

Quantification of JA and JA-Ile Levels

Leaf extracts were prepared essentially as described by Wang et al. (2007), with minor modifications. Briefly, 400 to 500 mg of leaf tissue was frozen in liquid N_2 and ground to a fine powder with a mortar and pestle. Dihydro-JA and ^{13}C-JA-Ile were added as internal standards for quantification of JA and JA-Ile, respectively. Following addition of 2.5 mL of ethyl acetate, homogenates were mixed and centrifuged at 12,000*g* for 10 min at 4°C. The supernatant was transferred to a new glass tube and the pellet was reextracted with 1 mL of ethyl acetate. The combined extracts were evaporated at 55°C under a stream of N_2 gas. The remaining residue was dissolved in 0.3 mL of 70% methanol/water (v/v) and filtered through a 0.2-μm polytetrafluoroethylene membrane (Millipore). Compounds in the resulting extract (5 μL of sample per injection) were separated on an UPLC BEH C18 column (1.7 μM, 2.1 × 50 mm) attached to an Acquity ultraperformance liquid chromatography system (Waters). A gradient of 0.15% aqueous formic acid (solvent A) and methanol (solvent B) was applied in a 3-min program with a mobile phase flow rate of 0.4 mL/min. The column, which was maintained at 50°C, was interfaced to a Quattro Premier XE tandem quadrupole mass spectrometer (Waters) equipped with electrospray ionization (negative mode). Transitions from deprotonated molecules to characteristic product ions were monitored for JA (*m/z* 209 > 59), dihydroJA (*m/z* 211 > 59), JA-Ile (*m/z* 322 > 130), and $^{13}C_6$-JA-Ile (*m/z* 328 > 136) using a 20-V collision cell potential for each ion. Peak areas were integrated, and the analytes were quantified based on standard curves generated by comparing analyte responses to the corresponding internal standard. Details regarding the performance of this method will be described elsewhere. Because this method does not distinguish JA-Ile from JA-Leu, values reported for JA-Ile represent the sum of JA-Ile plus JA-Leu (Wang et al., 2007). The level of JA-Leu in Arabidopsis seedlings is reported to be <25% of JA-Ile levels (Staswick and Tiryaki, 2004). ^{13}C-JA-Ile was synthesized by conjugation of (±)-JA (Sigma) to [$^{13}C_6$]-L-Ile (Cambridge Isotope Laboratories) as previously described (Kramell et al., 1988; Staswick and Tiryaki, 2004). For the experiment shown in Figure 6, total JA was extracted from 200 to 300 mg of leaf tissue using a vapor phase extraction method (Schmelz et al., 2004) and quantified by gas chromatography-mass spectrometry as previously described (Li et al., 2005).

RNA Gel-Blot Analysis

Primers used to amplify cDNA probes are described in Supplemental Table S2. The *VSP1* probe was described by Schilmiller et al. (2007). cDNAs were obtained by reverse transcription PCR of RNA isolated from wounded Arabidopsis (Col-0) leaves. Amplified cDNA fragments were cloned into vector pGEM-T Easy (Promega) and verified by DNA sequencing. These clones were used as templates for PCR reactions with gene-specific primers (Supplemental Table S2) to generate cDNA fragments that were used as probes in RNA blot hybridization experiments. The nucleotide identity

between all pairwise combinations of the 12 *JAZ* cDNAs ranged between 11% and 66%. The percent nucleotide identity between the most closely related pairs of *JAZ* genes is: *JAZ1* and *JAZ2*, 66%; *JAZ5* and *JAZ6*, 62%; and *JAZ7* and *JAZ8*, 60%. Thus, under the high stringency conditions used for hybridization experiments, full-length cDNA probes were assumed to be gene specific. RNA extraction and gel-blot analyses were performed as described previously (Li et al., 2002). Probed RNA blots were visualized with a phosphorimager and the signal intensities quantified with the Quantity One-4.2.2 program (Bio-Rad). Values for each time point were normalized to the *ACT8* loading control.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Phylogenetic tree of the Arabidopsis JAZ family.

Supplemental Table S1. *JAZ* genes are coexpressed with JA biosynthetic genes.

Supplemental Table S2. Oligonucleotide primers used in this study.

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