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Jasmonate Perception Regulates Jasmonate Biosynthesis and JA-Ile Metabolism: The Case of COI1 in Nicotiana attenuata

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CORONATINE INSENSITIVE 1 (COI1) is a well-known key player in processes downstream of jasmonic acid (JA) biosynthesis: silencing COI1 in Nicotiana attenuata (ir-coi1) makes plants insensitive to JA, prevents the up-regulation of JA-mediated defenses and decreases the plant’s resistance to herbivores and pathogens. In agreement with previous studies, we observed that regulation of several JA biosynthesis genes elicited by Manduca sexta oral secretions (OS) is COI1 dependent. In response to wounding and application of OS ir-coi1 plants accumulate 75% less JA compared with wild-type plants (WT), resembling JA levels found in plants silenced in the key enzyme in JA biosynthesis LIPOXYGENASE 3 (as-lox). However, while OS-elicited as-lox plants also accumulated lower levels of the JA-conjugate JA–isoleucine (JA–Ile) than did WT plants, JA–Ile accumulation in ir-coi1 was higher, prolonged and peaked with a delay of 30 min. In vivo substrate feeding experiments of N. attenuata demonstrate that the increased and prolonged JA–Ile accumulation pattern in ir-coi1 is not the result of altered substrate availability, i.e. of JA and/or Ile, but is due to an ~6-fold decrease in JA–Ile turnover. These results provide the first evidence for a second, novel regulatory feedback function of COI1 in enhancing JA–Ile turnover. Hence, in addition to its control over JA biosynthesis, COI1 might fine-tune the dynamics of the jasmonate response after induction by herbivore elicitors.

Keywords: CORONATINE INSENSITIVE1 — Jasmonic acid — Jasmonic acid–isoleucine — Nicotiana attenuata — Solanum lycopersicum.

Abbreviations: ACX1, ACYL-COENZYME A OXIDASE 1; ANOVA, analysis of variance; AOC, ALLENE OXIDE CYCLASE; AOS, ALLENE OXIDE SYNTHASE; as-lox, N. attenuata silenced in LOX3 via transformation with an antisense construct; COI1, CORONATINE INSENSITIVE 1; ir-coi1, N. attenuata silenced in COI1 via transformation with an inverted repeat construct; FDR, false discovery rate; JA, jasmonic acid; JA–Ile, jasmonic acid–isoleucine conjugate; JAR, JASMONIC ACID RESISTANT; LOX3, LIPOXYGENASE 3; MeJA, methyl jasmonate; OPDA, 12-oxophytodienoic acid; OPR3, OPDA REDUCTASE 3; OS, oral secretions collected from Manduca sexta larvae; 1RT–PCR, quantitative reverse transcription–PCR; TD, THREONINE DEAMINASE; WT, wild type.

The nucleotide sequences reported in this paper have been deposited in the database with the following accession numbers: NaACX1, EF467329, EF467330; NaOPR3, EF467331; NaAOC, EF467332.

Introduction

Herbivore attack elicits various defense responses in plants. These responses are preceded by a rapid and transient burst of jasmonic acid (JA) (Creelman et al. 1992, Farmer and Ryan 1992, Baldwin et al. 1994). The phytohormone, JA, as well as its precursors and derivatives are synthesized via the octadecanoid pathway (Creelman and Mullet 1997, Schaller et al. 2004, Wasternack 2007). According to the current model of the pathway, linolenic acid (18:3) is released from chloroplast membranes in response to different biotic and abiotic stimuli, and is oxygenated by a 13-lipoxygenase (13-LOX). The resulting 13(S)-hydroperoxylinolenic acid (13-HPOT) is dehydrated by allene oxide synthase (AOS) and cyclized by allene oxide cyclase (AOC), yielding 12-oxophytodienoic acid (9S, 13S-OPDA). The reduction of this cyclopentenone is catalyzed by OPDA reductase (OPR3) in the peroxisome, and followed by three cycles of β-oxidation resulting in 3R,7S-JA, which itself serves as a precursor for JA derivatives such as JA–amino acid conjugates or methyl jasmonate (MeJA). Several genes encoding enzymes involved in JA biosynthesis have been isolated and characterized in different plant species (reviewed by Wasternack 2007). Biological activity, i.e. the orchestration of defense responses to herbivore feeding (Halitschke and Baldwin 2004) and pathogen attack (Pozo et al. 2004), has been demonstrated not only for JA but also for some of its precursors (Stintzi et al. 2001) and derivatives (Staswick and Tiryaki 2004; Kang et al. 2006). The rapid increase of JA in response to wounding and herbivory is followed by the
up-regulation of genes involved in JA biosynthesis, indicating a positive feedback control of these genes (Sasaki et al. 2001, Reymond et al. 2004). While the kinetics and enzymatic reactions for JA accumulation in response to elicitation treatments have been well characterized in different plant species, it remains at present unclear how plants regulate the breakdown of JA and its modified forms (such as amino acid conjugates).

Several studies demonstrate that CORONATINE INSENSITIVE 1 (COI1) is involved in jasmonate perception and signaling (Xie et al. 1998, Chini et al. 2007, Thines et al. 2007). Induction of numerous herbivore- and wound-induced defense-related genes as well as the accumulation of defensive secondary metabolites were found to depend on COI1 and, consequently, several herbivores and pathogens perform better on plants that are deficient in this protein (Reymond et al. 2000, Ellis and Turner 2002, Li et al. 2004, Mewis et al. 2005, Paschold et al. 2007, Chung et al. 2008). The COI1 gene encodes an F-box protein that—as a subunit of the SCFCOI1 complex—targets proteins for degradation via ubiquitination (Devoto et al. 2002, Xu et al. 2002). Several members of a protein family called JAZ have been suggested to act as negative regulators of JA-induced transcription. Physical interaction of COI1 and JAZ1 in vitro has been demonstrated to be promoted by JA–isoleucine (JA-Ile). In addition, the COI1-mediated degradation of JAZ1 and JAI3 was shown to depend on jasmonate treatment, emphasizing the central role of COI1 within the octadecanoid signaling pathway.

In the model system comprising Nicotiana attenuata, a wild tobacco, and the tobacco hornworm Manduca sexta (Lepidoptera, Sphingidae), one of its major natural pests, the mechanisms of jasmonate signaling are well studied (Halitschke and Baldwin 2004, Kessler et al. 2004). Damage inflicted by M. sexta larvae elicits not only a rapid burst of free JA but also the accumulation of MeJA (von Dahl and Baldwin 2004) and several JA amino acid conjugates such as JA-Ile, JA-Trp, JA-Val and JA-Gln (Kang et al. 2006, Wang et al. 2007). The proteins JASMONIC ACID RESISTANT 4 and 6 (NaJAR4 and NaJAR6) were found to catalyze the in vivo conjugation of free JA to Ile. Accordingly, JA-Ile has been demonstrated to be essential for herbivore-induced defense signaling in N. attenuata (Kang et al. 2006, Wang et al. 2007, Wang et al. 2008). Importantly, supplying oral secretions (OS) of M. sexta larvae to the wounds of N. attenuata leaves recapitulates to a large extent the plant response to feeding by this insect, which is different from the wound response (McClosky and Baldwin 1997, Halitschke et al. 2003). Tomato (Solanum lycopersicum), another member of the Solanaceae family, also responds differentially to OS elicitation, whereas this response is only marginally present in the model species Arabidopsis thaliana.

To understand how N. attenuata’s COI1 affects herbivore-induced oxylipin biosynthesis and metabolism, we compared OS-elicited responses between WT plants and transgenic lines deficient in either JA signaling/perception (ir-coi1; Paschold et al. 2007) or JA biosynthesis (as-lox; Halitschke and Baldwin 2003).

Results

Analysis of transcript levels of JA biosynthesis genes in WT, ir-coi1 and as-lox plants

To evaluate how COI1 expression affects the regulation of JA biosynthesis genes in N. attenuata leaves, we analyzed their expression in WT, ir-coi1 and JA biosynthesis-deficient as-lox N. attenuata plants using: (i) oligo microarrays (Supplementary Fig. S1, Table S1) and (ii) quantitative real-time reverse transcription–PCR (qRT–PCR; Fig. S2). Plants were elicited by mechanical wounding followed by immediate application of M. sexta OS to the puncture wounds (hereafter this method is referred to as ‘OS elicitation’). Analysis of variance (ANOVA) of the microarray expression ratios across experiments revealed that the level of regulation of some of the JA biosynthesis was different between genotypes (Supplementary Table S1, Fig. S2). For example, both NaAOS and NaOPR3 were up-regulated to the same extent in WT and as-lox plants, but were significantly less up-regulated in ir-coi1 plants. However, the level of up-regulation of NaLOX3, a gene encoding a key enzyme in JA biosynthesis, was similar in WT and ir-coi1 plants but was much lower, as expected, in the as-locx line (Halitschke and Baldwin 2003). The OS-inducible THREONINE DEAMINASE (TD) gene (Kang et al. 2006) involved in the biosynthesis of isoleucine was normally induced after OS elicitation in as-lox and the WT, but not in ir-coi1 plants (Supplementary Table S1, Fig. S2). NaJAR4 and NaJAR6 are known to be up-regulated in response to wounding and OS elicitation in WT plants (Wang et al. 2007) as our microarray analysis confirmed. However, the level of up-regulation of these two genes was similar in ir-coi1 and as-locx plants 45 min after OS elicitation.

In addition to the octadecanoid pathway-related genes, several other genes showed genotype-specific differences in their early response to OS elicitation (Supplementary Table S1). For example, several photosynthesis-related genes (e.g. RUBISCO) were less down-regulated in ir-coi1 than in WT plants. In contrast, some genes functioning within the ethylene pathway [e.g. ACC oxidase (ACO)] the auxin pathway [e.g. indole-3-acetic acid amido synthetase (GHS)], the terpenoid biosynthesis pathway [e.g. 5-epi-aristolochene synthase (TEAS)] or the
phenylpropanoid pathway [e.g. phenylalanine ammonia lyase (PAL)] were less up-regulated in ir-coi1 mutants. Interestingly, hydoperoxide lyase (HPL) genes were not affected by silencing COI1, whereas several proteinase inhibitor (PIN) genes showed stronger up-regulation in ir-coi1 than in WT plants. The complete data set of the microarray analysis is presented in Supplementary Table S1.

The results of the microarray analysis were verified for selected octadecanoid pathway-related genes by means of qRT-PCR. A separate experiment was conducted to analyze the kinetics of transcript accumulation of selected genes within 3 h after OS elicitation (Fig. 1). Importantly, since these qRT–PCRs covered a time series that goes beyond 45 min, they show differences in the regulation of NaJAR4 and NaJAR6 in ir-coi1. At later time points, both NaJAR4 (Fig. 1d, ANOVA, genotype, $F_{1.46} = 21.674$, $P < 0.0001$) and NaJAR6 transcript levels (Fig. 1c, ANOVA, genotype, $F_{1.46} = 8.834$, $P < 0.0047$) were lower in ir-coi1 plants compared with the WT. Therefore, we extended our analysis of gene expression by means of qRT–PCR during a period of 3 h in the WT and ir-coi1 to other JA biosynthetic genes (Fig. 1a–c). This confirmed that silencing NaCOI1 does not impair the OS-elicited up-regulation of NaLOX3 (Fig. 1a, ANOVA, genotype, $F_{1.46} = 0.049$, $P > 0.05$). However, the induced levels of NaAOC (Fig. 1b, ANOVA, genotype, $F_{1.46} = 45.568$, $P < 0.0001$) and NaACX1 (Fig. 1c, ANOVA, genotype, $F_{1.46} = 22.127$, $P < 0.0001$) transcripts were lower in ir-coi1 than in WT plants also beyond 45 min, confirming the initial conclusions derived from the microarrays. Taken together, these results suggest that the activation of NaJAR4 and NaJAR6 genes is COI1 dependent; however, the effect of COI1 on the inducibility of NaJAR4 and NaJAR6 is less pronounced than the strong COI1 dependency of NaAOC and NaACX.

Oxylipin analysis in WT, ir-coi1 and as-lox N. attenuata and tomato plants

To examine the influence of COI1 on the biosynthesis of jasmonates, we analyzed the levels of free JA, OPDA and JA-Ile in OS-elicited N. attenuata WT, ir-coi1 and as-lox plants. For comparison, we also profiled OS-induced JA and JA-Ile levels in tomato (Solanum lycopersicum) WT and coi1 mutants. Free JA levels were reduced to approximately 25% of WT levels in ir-coi1 leaves (Fig. 2b, ANOVA, genotype $F_{2.78} = 55.274$, $P < 0.0001$, Bonferroni-corrected post hoc test: $P < 0.05$). Interestingly, the kinetics of free JA accumulation in ir-coi1 were similar to those of as-lox plants (Fig. 2b, Bonferroni-corrected post hoc test: $P > 0.05$). These results suggested that, similar to as-lox plants, ir-coi1 plants are impaired in JA biosynthesis. This conclusion was further substantiated by the 40–50% reduction in OPDA levels in ir-coi1 and as-lox compared with the WT after 30 min of OS-elicitation (Fig. 2a, ANOVA, genotype $F_{2.54} = 22.004$, $P < 0.0001$, Bonferroni-corrected post hoc test: $P < 0.05$). However, the JA-Ile accumulation in ir-coi1 was clearly delayed and more prolonged in ir-coi1 plants compared with the WT, and this is in strong contrast to as-lox plants that showed reduced JA-Ile levels during the entire course of the experiment (Fig. 2c, ANOVA, genotype $F_{2.78} = 110.002$, $P < 0.0001$; Bonferroni-corrected $P < 0.05$). From 0 to 30 min, the amounts of JA-Ile that accumulated in ir-coi1 plants were similar to those of as-lox and slightly lower than in the WT (Fig. 2c), suggesting that a reduced JA supply could be limiting JA-Ile formation at early time points in ir-coi1 and as-lox. After 60 min, leaves of ir-coi1 leaves had already accumulated ~30% more JA-Ile than WT leaves, and this difference also steadily increased after 90 and 120 min (Fig. 2c). This clearly shows that, unlike the situation in as-lox plants, JA-Ile accumulation in ir-coi1 is not impaired by a reduced JA supply in ir-coi1 leaves during that later phase of the response. Similarly, in tomato coi1 plants, the levels of free JA were reduced by ~2-fold compared with levels in the WT after induction (Fig. 3a, ANOVA, genotype: $F_{1.40} = 4.776$, $P = 0.035$) whereas JA-Ile levels were increased by ~2-fold over the course of the experiment (90 min) (Fig. 3b, ANOVA, genotype: $F_{1.40} = 33.181$, $P < 0.0001$). Taken together, in COI1 mutants JA accumulation is strongly reduced compared within WT plants, while JA-Ile accumulation appears to be increased and its pattern delayed and prolonged.

Isoleucine availability in ir-coi1 plants

To examine the possibility that the elevated JA-Ile levels in ir-coi1 plants resulted from elevated free Ile levels, we conducted a substrate feeding experiment using isotopically labeled Ile to measure the availability of endogenous Ile indirectly. By amended OS with $[^{13}C_6]$Ile and applying it to puncture wounds, we generated a second ($^{13}$C$_6$-labeled) Ile pool competing with the endogenous Ile pool during conjugation to JA at the wound site. This treatment resulted in a mixture of unlabeled JA-Ile and labeled JA-$[^{13}C_6]$Ile (Fig. 4a), and both compounds can be readily distinguished by mass spectrometry. We reasoned that if the endogenous Ile pool in ir-coi1 plants was larger than that in WT plants, the JA-$[^{13}C_6]$Ile/JA-Ile ratio would be smaller in ir-coi1 plants than in WT plants, and vice versa. Treatment with labeled $[^{13}C_6]$Ile indeed decreased the absolute amount of unlabeled JA-Ile in all genotypes (Fig. 4b). The absolute levels of unlabeled JA-Ile (Fig. 4b, ANOVA, $F_{2.12} = 14.421$, $P = 0.0006$) and labeled JA-$[^{13}C_6]$Ile (Fig. 4c, ANOVA, $F_{2.11} = 15.295$, $P = 0.0007$) were similar between WT and ir-coi1 plants (Bonferroni-corrected post hoc test: $P > 0.05$) and lower in as-lox plants ($P < 0.05$) as expected. The ratio of JA-$[^{13}C_6]$Ile and JA-Ile...
Fig. 1  OS-induced expression pattern of oxylipin biosynthesis genes in WT (filled squares) and ir-coi1 (filled triangles) N. attenuata plants. The expression of different genes involved in JA biosynthesis (LOX3, AOS and ACX) and JA modification (JAR4 and JAR6) was measured by qRT–PCR after OS elicitation. Leaves from rosette-stage N. attenuata plants were wounded and immediately treated with OS. Total RNA was extracted and utilized to perform qRT–PCR. Quantification of transcript abundance is described in detail in Materials and Methods.
did not differ between WT and as-lox plants (Fig. 4d, ANOVA, $F_{2,11} = 8.966$, $P = 0.0049$, Bonferroni-corrected post hoc test, $P < 0.05$). However, the JA-[13C6]Ile/JA-Ile ratio was larger in ir-coi1 plants ($P = 0.0281$) than in WT plants, indicating a decreased but certainly not increased endogenous Ile pool in ir-coi1 leaves.

Analysis of JA-Ile accumulation and metabolism

Clearly, the pool of Ile in ir-coi1 (Fig. 4) did not account for the plant's increased and prolonged JA-Ile accumulation. Hence, we tested whether this phenotype is caused by (i) changes in the availability of other substrates or (ii) decreased JA-Ile turnover. Therefore, we conducted a series of additional OS elicitation experiments on WT, ir-coi1 and as-lox plants in which we exogenously supplied substrates together with the OS at the wound site.
In the first experiment, the OS was amended with either 0.625 m mol Ile (w + OS + Ile), 0.625 m mol JA (w + OS + JA) or both mixed together (w + OS + Ile + JA) (Fig. 5a). The amounts of JA-Ile were quantified 1 and 2 h after elicitation, time points which correspond to the peak of JA-Ile accumulation in ir-coi1 and its return to almost basal levels in WT, respectively (Fig. 2c). In accordance with the previous [13C6]Ile experiment, treating the wounds with OS and Ile did not increase the amounts of JA-Ile in any of the genotypes after either 1 h (Fig. 5b, ANOVA, treatment $F_{3,43} = 64.249$, $P < 0.0001$, Bonferroni-corrected post hoc test, $P > 0.05$) or 2 h (Fig. 5c, ANOVA, treatment: $F_{3,43} = 118.855$, $P < 0.0001$, Bonferroni-corrected post hoc test, $P > 0.05$) compared with the (w + OS + water) controls. In contrast, when OS and JA were applied to the wounds, the amount of JA-Ile significantly increased in all genotypes tested (Bonferroni-corrected post hoc test, $P < 0.0001$) reaching the highest levels in ir-coi1 after 2 h (Fig. 5c). These results suggested that JAR activity, despite lower transcript levels in ir-coi1, is not limiting in any of the genotypes we tested. Moreover, when OS, Ile and JA were supplied, ir-coi1 plants produced significantly more JA-Ile than did WT or as-lox plants after 1 h ($\sim 40\%$, Fig. 5b, ANOVA, genotype $F_{2,44} = 9.984$, $P = 0.0003$) and 2 h of the treatment ($\sim 100\%$, Fig. 5c, ANOVA, genotype: $F_{2,43} = 54.003$, $P < 0.0001$, Bonferroni-corrected post hoc test, $P > 0.05$) compared with the (w + OS + water) controls.
post hoc test: $P<0.0001$). Thus, the experiments confirmed that substrate availability (most probably JA) is limiting JA biosynthesis in ir-coi1 and therefore suggest that increased JA-Ile accumulation in ir-coi1 is due to a lower JA-Ile turnover.

To investigate if lower JA-Ile turnover explains its accumulation pattern in ir-coi1, we applied physiological amounts of JA-[13C6]Ile to OS-treated wounds of WT and ir-coi1 plants. The levels of the substance were quantified over a 3 h period. In the treated tissue, the amounts of JA-[13C6]Ile did not change during the first 30 min after application and therefore we analyzed of the turnover of JA-[13C6]Ile at selected intervals between 30 min (100%) and 3 h after application (Fig. 6). The results indicated that the initial turnover rates (time 30–60 min) of JA-Ile in ir-coi1 and WT leaves were approximately 50 and 330 ng h$^{-1}$/C0, respectively (~6-fold). Moreover, 90 min after application, the initial JA-[13C6]Ile amounts were reduced by 5% in ir-coi1 but by 35% in WT plants (Fig 6). Thus, the applied JA-[13C6]Ile decreased faster in WT than in ir-coi1 plants. Quantification of JA-[13C6]Ile in the adjacent untreated tissue of the treated leaf revealed that at all times, and for both genotypes, the amounts were <5% of the amounts in the treated tissue (data not shown), indicating that the loss of JA-[13C6]Ile could not be explained by differences in transport and/or diffusion. The results strongly suggest that the delayed and more prolonged accumulation of JA-Ile in ir-coi1 lines is caused by reduced turnover/modification of JA-Ile in this genotype.

**Fig. 5** Analysis of JA-Ile accumulation in ir-coi1 plants. (a) The JA–amino acid conjugate, JA-Ile, is the product of a conjugation reaction between JA and Ile, which is mediated by JAR4 and JAR6. Rosette-stage plants were wounded and treated with OS amended with excess amounts (0.625 nmol) of one of the substrates (w + OS + JA and w + OS + Ile) or both (w + OS + JA + Ile). JA-Ile accumulation was analyzed (b) 1 h and (c) 2 h after treatment.
Discussion

In this study, we aimed to understand the regulatory activity of the F-box protein COI1 on oxylipin biosynthesis, metabolism and gene expression in N. attenuata after OS elicitation. In order to separate the effect which COI1 has on the JA biosynthesis pathway from its effect on JA signal transduction, we compared the OS-elicited responses in WT, ir-coil and as-lox N. attenuata plants. The analysis showed that both ir-coil and as-lox have similar low levels of induced JA compared with the WT. However, in contrast to as-lox, the pattern of JA-Ile accumulation in ir-coil was increased, but also delayed and more prolonged compared with the WT, resulting in higher total amounts after 60 min of OS elicitation (Fig. 2c). Similar JA and JA-Ile patterns were found in coil tomato (S. lycopersicum) compared with the corresponding WT.

Compared with the leaves of WT, ir-coil leaves accumulated slightly reduced amounts of JA-Ile during the early time points, i.e. between 15 and 45 min after OS elicitation, suggesting that its reduced JA accumulation was responsible for the approximately 30 min delay in the JA-Ile accumulation peak (Fig. 2c). The fact that gene expression of JAR4/6 was lower overall in ir-coil than in WT suggested that the plants did not compensate for their low levels of JA by increasing JAR expression and thereby activity. Moreover, the comparison of ir-coil lines with as-lox lines showed that while both genotypes had similar low levels of JA, only ir-coil prolonged the accumulation of JA-Ile. Feeding JA exogenously to the OS-elicited leaves of N. attenuata increased the amount of JA-Ile in all genotypes tested, but this effect was most prominent in ir-coil plants after 2 h of the treatment. In addition, by feeding [13C6]Ile we could show that the endogenous Ile pool in coil tomato (S. lycopersicum) was lower overall in ir-coil plants. Thus we could rule out unambiguously that increased Ile levels caused the increased JA-Ile levels in ir-coil plants. Finally, the analysis of JA-Ile turnover rates in WT and ir-coil leaves after OS elicitation indicated that this process is retarded in ir-coil compared with WT plants, suggesting that the differential turnover of JA-Ile is the major determinant of JA-Ile accumulation in ir-coil. Taken together, the results strongly suggest that COI1 has a stimulatory function in JA-Ile catabolism—in addition to its stimulatory role—providing positive feedback on JA biosynthesis.

Previous transcriptional profiling studies performed on Arabidopsis coil-1 already demonstrated the requirement of COI1 for the transcriptional induction of JA biosynthesis genes (Reymond et al. 2000, Sasaki et al. 2001, Devoto et al. 2005, Taki et al. 2005). An interesting difference between Arabidopsis coil-1 and N. attenuata ir-coil is the COI1-dependent regulation of the first committed enzyme in JA biosynthesis, AtLOX2 and NaLOX3, respectively. These two enzymes have a well-described role in JA biosynthesis, and silencing them has strong effects on JA biogenesis in both species (Bell et al. 1995, Halitschke and Baldwin 2003). In Arabidopsis coil-1, AtLOX2 transcript levels were several-fold lower in unwounded leaves, and are not increased after wounding, compared with the WT (Reymond et al. 2004, Devoto et al. 2005). In contrast, NaLOX3 mRNA levels in ir-coil leaves were similar to those in non-elicited and OS-elicited WT leaves. These results indicate that NaLOX3 gene expression in N. attenuata may be COI1 independent. However, since COI1 expression in ir-coil lines was not eliminated completely, residual COI1 expression in these plants might be sufficient to activate LOX3 expression. Similar to Arabidopsis coil-1, the majority of oxylipin biosynthesis genes analyzed in N. attenuata (NaAOS, NaAOC, NaOPR3 and NaACX1) appeared to be COI1 dependent, but the differences in their induction suggest a differential quantitative dependence on COI1 levels. As previously proposed, the positive feedback of COI1 on the expression of JA biosynthesis genes might serve to support JA defenses during continuous herbivore feeding or pathogen attack (Reymond et al. 2000, Sasaki et al. 2001, Devoto et al. 2005). Also in accordance with these previous studies, the early response of genes involved in other biological processes indicates that COI1-mediated signaling is not solely involved in jasmonate responses. For example, the COI1-dependent up-regulation of genes encoding enzymes involved in the phenylpropanoid pathway (e.g. PAL) has also been reported from studies on Arabidopsis coil-1 mutants (Reymond et al. 2004, Devoto et al. 2005).

Our novel finding that COI1 also positively regulates JA-Ile turnover presumably reflects its tight control over the dynamics of the JA response which might be established by directly or indirectly controlling the transcription of JA-Ile metabolizing enzymes. In contrast to other phytohormones such as auxins or gibberellins, our knowledge of the catabolism of active jasmonates is still limited. Although several derivatives of JA such as 12-OH-JA and O-glucosyl-JA have been identified (Miersch et al. 2008), the fate of JA and most of the enzymes mediating this process remains to be elucidated. In general, to maintain the effectiveness of induced responses, the removal of active signals is as important as their production, and in order to understand the kinetics of plant signaling compounds both of these processes should be carefully analyzed.

Materials and Methods

Plant growth conditions and rearing M. sexta larvae

We used seeds of the 14th generation of an inbred line of N. attenuata Torr. Ex Watts as the wild-type genotype (WT) in all experiments. In addition to WT plants, we used ir-coil plants (Paschold et al. 2007) and as-lox plants (Halitschke and
Baldwin 2003). Seed germination and plant growth were conducted as previously described (Krügel et al. 2002). The tomato (S. lycopersicum cv Castlemart) WT and coi1 (Li et al. 2004) seeds were kindly provided by Gregg Howe (Michigan State University, USA) and were pre-germinated on water-saturated paper. Homozygous coi1 seedlings were selected on filter paper saturated with 1 mM MeJA according to the procedure described by Li et al. (2004). After 1 week, the selected seedlings were transferred to soil and grown under the same conditions as N. attenata plants. Manduca sexta larvae were reared as previously described (Paschold et al. 2007) and their OS were collected from third to fourth instar larvae as previously described (McCloud and Baldwin 1997).

**JA and JA-Ile accumulation—kinetics experiments**

Rosette-stage N. attenata plants were puncture wounded and immediately treated with 20μl of 1/5 (v/v) OS/water (OS elicitation). This dilution was used in all experiments unless stated otherwise. Leaf tissue was harvested 15, 30, 60, 90, 120 and 180 min after OS elicitation, and control samples were taken simultaneously from untreated plants. To analyze phytohormone levels in tomato plants, three fully developed leaflets of 4-week-old plants were wounded and treated with 20μl of 1/5 (v/v) water-diluted OS collected from M. sexta larvae raised on tomato WT plants. Leaves were harvested at 30, 60 and 90 min after elicitation, while control samples were taken just before the elicitation treatment.

**Substrate feeding experiments**

All substrate feeding experiments were conducted on rosette-stage plants. To analyze the availability of Ile in WT, ax-lox and ir-coi1 plants, fully developed leaves were elicited by first wounding them with a pattern wheel and applying OS that contained 625μmol [13C6]Ile [dissolved in 30% (v/v) ethanol/water, Cambridge Isotope Laboratories, Inc., http://www.isotope.com] to the wounds while control plants were wounded and treated with similarly diluted OS [diluted in 30% (v/v) ethanol/water]. To analyze the accumulation of JA-Ile under non-limiting substrate conditions, fully developed leaves were wounded and treated with OS that contained 0.625μmol L-isoleucine (Sigma, http://www.sigmaaldrich.com). 0.625μmol JA or both [each dissolved in 30% (v/v) ethanol/water]. Control plants were wounded and treated with OS that had been diluted with 30% (v/v) ethanol/water. Leaves were harvested 1 h after the treatment. For the JA-Ile turnover experiment, JA-[13C6]Ile synthesis was adapted from the method reported by Koch et al. (1999) using [13C6]Ile-isoleucine (98%; Cambridge Isotope Laboratories, http://www.isotope.com). Purification was achieved by solid phase elution. Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS; Varian 1200L LC-MS/MS; Varian; http://www.varianinc.com) analysis showed no detectable free JA and the yield of the reaction was 32.8 mg (82%). To analyze the turnover of JA-Ile, a fully developed rosette leaf was divided into three sections and only the middle section was carefully wounded and treated with OS. After 30 min, 500 ng of JA-[13C6]Ile [dissolved in 0.01% (v/v) aqueous Tween-20] were applied to the wounded area. The treated part and the untreated distal parts of the leaf were harvested after 30, 60, 90, 120 and 180 min, and JA-[13C6]Ile was analyzed as indicated below.

**Phytohormone analysis**

Phytohormones were extracted as previously described (Wang et al. 2007). Each sample was spiked with 200 ng of [13C6]JA and p-coumaric acid (Sigma-Aldrich, Munich, Germany). Phytohormones were quantified using the Varian 1200L LC-MS/ MS system as described (Wang et al. 2007).

**Analysis of transcript levels by microarrays and qRT–PCR**

To analyze changes in gene expression by microarrays, fully developed leaves of rosette-stage WT, ax-lox and ir-coi1 plants were puncture wounded and treated with OS (n = 9 per genotype). Control plants were left untreated (n = 9 per genotype). Leaf material from all plants was harvested 45 min after elicitation and immediately frozen in liquid nitrogen. The leaf tissue of each individual sample was ground under liquid nitrogen and RNA was extracted using TRI reagent as previously described (Paschold et al. 2007). A 150 μg aliquot of total RNA from three individual control samples was pooled. Three induced samples were pooled identically. From each pool mRNA was isolated, reverse-transcribed and labeled as previously described (Halitschke et al. 2003). Induced samples were labeled with Cy3 and hybridized against control samples labeled with Cy5. For all hybridizations, three independent microarray experiments were performed. We used a custom-made 5.6K microarray consisting of 1,400 50mer oligonucleotides all spotted four times (Wang et al. 2007). Spot intensities for Cy3 and Cy5 were extracted from image files using the AIDA software (Raytest, http://www.raytest.com/). Raw signal intensities were local background subtracted and LOWESS-normalized using MIDAS (Saeed et al. 2003, see also http://www.tm4.org/). Spots with intensity <1.5× the local background were set to zero. For statistical analysis, normalized intensity values were 2Log transformed (in Supplementary Table S1 the back-transformed ratios are shown). To analyze the three replicate microarrays (Supplementary Table S1), a nested ANOVA was performed and the obtained P-values for all clones, denoting their significance of regulation, were adjusted for multiple testing using Benjamini and Hochberg’s step-up procedure for controlling the false discovery rate (FDR; Benjamini and Hochberg 1995). To compare the level of regulation of individual JA biosynthesis genes between genotypes, we used the three ratios of significantly regulated clones of each of the replicated hybridizations of WT, as-lox3 and ir-coi1, respectively. We performed the across-slide analysis by means of a one-way ANOVA and a post hoc test (Fisher’s LSD). The collection of P-values obtained from this analysis was also FDR-adjusted. Hence, the outcome of this analysis revealed the differences in the expression ratios of selected genes between the genotypes.

Quantitative real-time PCR (TaqMan®) was performed using samples from a kinetic experiment (n = 5 per time point, treatment and genotype). cDNA was synthesized from 150 ng of RNA using MultiScribe™ reverse transcriptase (Applied Biosystems, http://www.appliedbiosystems.com). Quantitative real-time PCR (ABI PRISM™7000, Applied Biosystems) was conducted using the qPCR™ core reagent kit (Eurogentec, http://www.eurogentec.be) and gene-specific TaqMan® primer pairs for NaLOX3 (Halitschke and Baldwin 2003), JAR4 (Kang et al. 2006), JAR6 (Wang et al. 2007), NaAOS (Halitschke et al. 2004) and NaTD (Kang et al. 2006). Transcript amounts were quantified based on a standard curve generated with cDNA of known concentration. The expression levels of NaOPR3, NaAO and NaACX1 mRNAs were analyzed by qRT–PCR using a SYBR® green assay. cDNA was synthesized as described above. Specific primers for NaOPR3 (FP, 5‘-AATGAGTTGGAGAGTTTTG-3’; RP, 5‘-AGGTTGTTG TAAAGAGTCTTT-3’), NaAO (FP, 5‘-ACCCTAAGCCCTC-3’ and RP, 5‘-TGGAAACCCCAACC-3’), and NaACX1 (FP, 5‘-GAATGTCGGAGATTTGCT-3’; RP, 5‘-TAGGTTTCTATGACGTT-3’; and the qPCR Core
kit for SYBR® Green I (Eurogentec, http://www.eurogentec.be) were used following the manufacturer’s instructions. NopR3, NaACX1 and NaAOC have been isolated within this study and their function is assumed based on their homology to characterized genes in other species. Transcript abundance was calculated and expressed as described above.

**Statistical analyses**

The data were analyzed with StatView (SAS Institute, http://www.sas.com) and SPSS (SPSS, http://www.spss.com), and are expressed as the mean (±SE). In the case of non-homogenous variances, data were (log + 1)-transformed.

**Supplementary data**

Supplementary data mentioned in the article is available at *Plant and Cell Physiology* online.

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**References**


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