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COI1-regulated hydroxylation of jasmonoyl-L-isoleucine impairs *Nicotiana attenuata*'s resistance to the generalist herbivore *Spodoptera litura*

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ABSTRACT

The phytohormone jasmonoyl-L-isoleucine (JA-Ile) is well known as the key signaling molecule that elicits plant defense responses after insect herbivory. Oxidation, which is catalyzed by the cytochrome P450s of the CYP94 family, is thought to be one of the main catabolic pathways of JA-Ile. In this study, we identified four *CYP94B3* homologues in the wild tobacco plant *Nicotiana attenuata*. Individually silencing the four homologues revealed that *NaCYP94B3 like-1* and *NaCYP94B3 like-2*, but not *NaCYP94B3 like-3* and *NaCYP94B3 like-4*, are involved in the C-12-hydroxylation of JA-Ile. Simultaneously silencing three of *NaCYP94B3 like* genes, *NaCYP94B3 like-1*, *2*, and *4* in the VIGS-NaCYP94B3s plants doubled herbivory-induced JA-Ile levels and greatly enhanced plant resistance to the generalist insect herbivore, *Spodoptera litura.* The poor larval performance was strongly correlated with the high concentrations of several JA-Ile-dependent direct defense metabolites in VIGS-NaCYP94B3s plants. Furthermore, we show that the abundance of 12-hydroxy-JA-Ile was dependent on JA-Ile levels as well as COI1, the receptor of JA-Ile. COI1 appeared to transcriptionally control *NaCYP94B3 like-1* and *2* and thus regulates the catabolism of its own ligand molecule, JA-Ile. These results highlight the important role of JA-Ile degradation in jasmonate homeostasis and provide new insight into the feedback regulation of JA-Ile catabolism. Given that silencing these *CYP94* genes did not detectably alter plant growth and highly increased plant defense levels, we propose that *CYP94B3* genes can be potential targets for genetic improvement of herbivore-resistant crops.

KEYWORDS: JA-Ile, catabolism, defense, plant-insect interaction, cytochrome P450, *Nicotiana attenuata*, *Spodoptera litura*

INTRODUCTION

During a long-term co-evolutionary process, plants have evolved sophisticated and effective mechanisms to defend themselves against herbivore attack. Herbivory induces the production of many defenses including toxic secondary metabolites and defensive proteins in plants, which counteract herbivore attack. Among these defenses, many are positively regulated by the jasmonates[1, 2]. Jasmonates commonly refer to jasmonic acid (JA), as well as to its precursors and metabolites derived from the oxylipin biosynthesis pathway. During herbivore feeding, the wounding rapidly elicits plants JA synthesis, and in some plants (but not all), certain components in herbivore oral secretions, such as fatty acid-amino acid conjugates (FACs), can be recognized and thus further amplifies the wound-induced JA[3].

The biosynthesis of JA starts with the release of linolenic acid from chloroplast membranes. Following reactions catalyzed by 13-LIPOXYGENASE (13-LOX), ALLENE-OXIDE SYNTHASE (AOS), and ALLEN-OXIDE CYCLASE (AOC), 12-oxo-phytodienoic acid (OPDA) is synthesized and then transported to the peroxisomes. Here, OPDA is converted to JA by OPDA REDUCTASE (OPR) and a β-oxidase complex[4]. JA can further be conjugated with amino acids, particularly Ile (forming JA-Ile), a reaction catalyzed by JASMONATE-RESISTANT1 (JAR1)[5]. The receptor of the bioactive hormone JA-Ile is a F-box protein, CORONATINE-INSENSITIVE1 (COI1)[6], which is a part of an E3 ubiquitin ligase SCF^{COI1} complex[7 8 9]. Briefly, in the absence of JA-Ile, the JASMONATE ZIM-DOMAIN (JAZ) repressor proteins bind MYC2, an important transcript factor in JA signaling, and repress MYC2 activity. In response to internal or external cues, JA-Ile is synthesized by JAR1 and specifically binds to the JAZ-COI1 co-receptor complex. This binding event facilitates the ubiquitination of JAZs by SCF$^{\mathrm{COI1}}$ and leads to degradation of JAZs through the 26S proteasome-mediated pathway. JA-Ile functions as a signaling molecule and induces the production of many defenses including secondary metabolites and proteins[10, 11], such as glucosinolates[12], nicotine [13], trypsin proteinase inhibitors (TPIs)[14], and 17-hydroxygeranyllinalool diterpenoid glycosides (HGL-DTGs)[15].

Although highly increased JA is required to deploy defense against herbivores, the catabolism of jasmonates is also necessary for balancing defense and growth[16, 17]. Exogenously applied jasmonates can enhanced defense but compromise plant fitness [18]. Similarly, when Arabidopsis was repeatedly wounded, its growth was highly stunted, due to the wound-induced accumulation of JA [17]. Previously, two JA-Ile catabolic pathways have been reported. One is mediated by JASMONOYL-L-ISOLEUCINE HYDROLASE 1 (JIH1), which was first identified in the wild tobacco *Nicotiana attenuata*, catalyzing direct hydrolysis of JA-Ile to JA. The *JIH1* gene-silenced plants have increased JA-Ile levels after simulated herbivory and enhanced resistance to the specialist *Manduca sexta* and generalist *Spodoptera littoralis* insects, compared with wild-type (WT) plants[19]. The other is cytochrome P450 CYP94 subfamily-mediated hydroxylation and carboxylation of JA-Ile. Three members of CYP94 subfamily, CYP94B1, CYP94B3 and CYP94C1, initially characterized in Arabidopsis, sequentially oxidize JA-Ile to hydroxyl-(12OH-JA-Ile) and dicarboxy-(12COOH-JA-Ile) derivatives[20, 21]. The CYP94 family genes have been found in most land plants, including non-vascular plants (e.g., *Physcomitrella patens*) [22]. CYP94B3 and CYP94B1 coordinately govern the majority (>95%) of 12-hydroxylation of JA-Ile in wounded arabidopsis leaves[23]. Compared with JA-Ile, 12OH-JA-Ile is less active in promoting COI1-JAZ interactions, although this oxidized derivative maintains some activity as a receptor ligand[20]; in contrast, 12COOH-JA-Ile is inactive in promoting COI1-JAZ interactions[23, 24]. CYP94B3- and CYP94B1-overexpressing plants displayed phenotypes indicative of JA-Ile deficiency, including defects in male fertility, resistance to jasmonate-induced growth inhibition[20, 23]. The functions of some CYP94s in pathogen resistance have been demonstrated. It was reported that *cyp94b3* mutants are more susceptible to the hemi-biotrophic bacterial pathogen *Pseudomonas syringae* pv *tomato* DC3000, suggesting the possibility that increased JA-Ile content compromises host resistance to this pathogen by suppressing the salicylate-mediated immunity[25]. In addition, *CYP94B3*-overexpressing plants showed impaired resistance to the necrotrophic pathogen *Botrytis cinerea*, most likely due to reduced JA-Ile levels, and thus strongly impaired defense gene induction[24]. Despite causing JA-Ile over-accumulation due to impaired oxidation, *cyp94b1b3c1* triple mutants had negligible changes in resistance to *B. cinerea*, which was associated with enhanced *JAZ* repressor transcript levels[24]. However, the roles and consequences of JA-Ile oxidation in plant-herbivore interaction remain largely unexplored.

N. attenuata is an annual wild tobacco that inhabits the western North America. It occurs ephemerally in large populations after perceiving certain unknown germination cues in wood smoke generated by wild fires. As a consequence of this particular germination behavior, *N. attenuata* chases fires in ecological time and has evolved to grow in habitats that share most of the same selection pressures that agricultural plants face: a large unpredictable herbivore community. Many molecular tools, including transformation systems and virus-induced gene silencing (VIGS), have been well developed. These all make *N. attenuata* a very attractive system for studying plant resistance to insects[18].

Here, we identified four *N. attenuata CYP94B3 like* genes and examined their function in plant defense against the lepidopteran herbivore *Spodoptera litura*. Three of four *CYP94B3 like* genes were transcriptionally induced by wounding and simulated herbivore attack and their independent silencing revealed that two had JA-Ile hydroxylation activity. These two homologues were simultaneously silenced and the silenced plants were more resistant to the generalist herbivore *S. litura* due to elevated JA-Ile levels, which in turn elicited the accumulation of defense-related metabolites. We further found that JA-Ile hydroxylation is transcriptionally regulated by the receptor COI1. The impaired catabolism of JA-Ile in *COI1* knock-down plants showed a prolonged JA-Ile accumulation pattern. These results reveal the critical role of *CYP94B3 like* genes in maintaining JA-Ile homeostasis, and thus are an important part of the plant defense network against herbivore attack. We also propose that these genes could be new targets of genetic improvement of insect resistant crops using breeding or transgene technology.

MATERIALS AND METHODS

Plant growth and *Spodoptera litura* feeding assay

N. attenuata seeds that were originally collected from its native habitat in Great Basin desert, Utah (USA), and had been inbred for 31 generations were used for the experiments. Seed germination and plant growth were conducted as described previously[26]. *iraoc*[27], *irjar4/6*[10], and *ircoi1*[28] plants were generated as described. Eggs of *Spodoptera litura* were obtained from Beijing Genralpest Company (http://genralpest.b2b.hc360.com/) and kept in a growth chamber at 26℃/16 h light, 24℃/8 h darkness until the larvae hatched. Eighteen to 21 freshly hatched larvae were placed individually on different plants, and larval masses were measured on day 11 and 15.

Isolation of full-length cDNAs of *CYP94B3* homologues in *N. attenuata*

The protein sequences of AtCYP94B3 and AtCYP94B1were used as queries to search the *N. attenuata* genomic sequence database (Baldwin et al., unpublished data). Based on the sequence similarities, the putative full length cDNAs of *NaCYP94B3 like-1, -2, -3, -4* were amplified and sequenced (GenBank accession numbers KT355399, KT355400, KT355401, and KT355400, respectively). The primers used are listed in Table S1.

Jasmonate syntheses and plant treatments

JA-Ile and JA-$^{13}C_6$-Ile were synthesized using JA, Ile, and $^{13}C_6$-Ile[29]. 12OH-JA-Ile was synthesized as following: to the solution of racemic mixture 2-(2-(5-hydroxypent-3-enyl)-3-oxocyclopentyl) acetic acid (Olchemim, Cezch), L-isoleucine-2-d_1 (C/D/N Isotopes Inc., https://www.cdnisotopes.com) and O-(7-aza-1H-benzotriazol-1-yl)-N, N, N', N'-tetramethyluronium hexafluorophosphate in tetrahydrofuran was triethylamine added slowly. Stirring was continued for 5 h at room temperature. The reaction mixture was concentrated in a vacuum to produce a brown-yellow oil. The resulting oil was purified by chromatography on silica gel with petroleum chloroform–methanol (5:1). The eluent yielded colorless 12OH-JA-Ile. Chemicals without specific description were all obtained from Sigma (http://www.sigmaaldrich.com).

S. litura larval oral secretions and regurgitants (OS) were collected with a pipette and stored at -20 °C. For ‘W+W’, ‘W+OS’, ‘W+OS+JA’, or ‘W+OS+JA-Ile’ treatments, the leaf growing at node +1, which is one leaf position older than the source-sink transition leaf, was wounded by rolling a fabric pattern wheel over the leaf surface to produce standardized puncture wounds, and immediately after wounding, 20 µL of deionized H_2O (W+W), OS at a 1:5 dilution (W+OS), 1:5 diluted OS containing 10 µg of JA (W+OS+JA), or 1:5 diluted OS containing 10 µg of JA-Ile (W+OS+JA-Ile), were applied to wounds.

Quantitative real-time PCR assay (qPCR)

Total RNA was extracted from ground leaf samples using the TRIzol reagent (Invitrogen) following manufacturer’s instructions. cDNA was synthesized from 500 ng of total RNA with RevertAid™ H Minus Reverse Transcriptase (Thermo Scientific). Quantitative PCRs were performed by SYBR-green analysis using *N. attenuata actin2* gene as an internal standard, with the protocol described before[30].

Generation and Characterization of VIGS Plants

PCR was used to generate fragments of *NaCYP94B3 like-1*, *-2*, *-3*, and *-4* from *N. attenuata*. Primers used for VIGS construction are listed in Table S1. The obtained PCR fragments were first cloned into pMD18-T vector (Takara) and sequenced. Plasmids with correct insertion were digested with BamH I and Hind III; and the resulting fragments were cloned into the pTV00 vector digested with the same enzymes. The *A. tumefaciens* (strain GV3101)–mediated transformation procedure for VIGS was described previously[31]. To monitor the progress of VIGS, we simultaneously silenced another set of plants with a construct specific for *phytoene desaturase* (*NaPDS*), resulting in visible bleaching of green tissues. When the leaves of *NaPDS*–silenced plants sufficiently bleached (~6 weeks after germination), leaves of *NaCYP94B3* likes-silenced (VIGS-NaCYP94B3s), and empty vector–inoculated (VIGS-EV) plants were used for experiments.

Jasmonate analyses using HPLC-MS/MS

Approx. 200 mg of leaf tissue of each sample were ground in liquid nitrogen. Phytohormones were extracted with 1 mL of ethyl acetate spiked with 100 ng of D_5-JA (2, 4, 4-d_3; acetyl-2, 2-d_2, C/D/N Isotopes Inc.), 20 ng of JA-$^{13}C_6$-Ile as the internal standards. After vortexing for 10 min, the organic phase was obtained by centrifugation at 16100 g for 15 min at 4°C. Samples were evaporated to dryness in a vacuum concentrator (Eppendorf) under reduced pressure at 30°C. Leaf samples were then resuspended in 600 μL of methanol: water (70: 30, v/v), and centrifuged at 16100 g for 15 min at 4°C. The resulting supernatants were directly injected in to a Shimadzu 8040 HPLC-MS/MS system. Separation was achieved on a Shimadzu pack XR-ODS column (2.0mmi.d, 75 mm, Shimadzu). Formic acid (0.05%) and ammonium formate (5 mM) in water (A) and methanol (B) were employed as the mobile phases: 0 to 0.01 min, 20% B; 0.01 to 8.0 min, 20% to 95% B; 8.0 to 8.1 min, 20% B. The mobile phase flow rate was 0.3 mL min^{-1}. The column temperature was maintained at 50 °C. ESI was operated in the negative ionization mode. The instrument parameters were optimized by infusion experiments with pure standards. The ion spray voltage was maintained at −4,500 eV. The heating block temperature was set at 500 °C. The DL temperature was set at 300°C. Nebulizing gas was set at 3 L min^{-1}, drying gas flow at 15 L min^{-1}, and collision gas at 230 kPa. Multiple reaction monitoring was used to monitor analytic parent ions to product ions. Both Q1 and Q3 quadrupoles were maintained at unit resolution. LabSolutions main software

(Shimadzu) was used for data acquisition and processing. Linearity in ionization efficiencies was verified by analyzing dilution series of standard mixtures. Quantities of phytohormones were calculated according to the ratios of their ion intensities and according to their respective internal standards. For 12OH-JA-Ile, JA-$^{13}C_6$-Ile was used as the internal standard. All quantifications were corrected according to the sample dilution and leaf masses used. All extractions were performed using 3-5 biological replicates.

Analysis of direct defense traits

Nicotine, chlorogenic acid, and diterpene glycosides were analyzed by HPLC as described previously [32], except that 100 mg of tissue were used. After centrifuging for 10 min at 13200 *g*, the supernatants were used for analysis. TPI activity was analyzed using a radial diffusion activity assay as described[33].

RESULTS

Cloning of *AtCYP94B3* homologues from *N. attenuata*

To clone the homologues of Arabidopsis AtCYP94B3 and AtCYP94B1, two enzymes hydroxylating JA-Ile[20, 23], we searched the *N. attenuata* genomic sequence database (Baldwin et al., unpublished data) using the protein sequences of AtCYP94B3 and AtCYP94B1 as queries. Totally four candidates, named NaCYP94B3 like-1, -2, -3, and -4, respectively, were obtained. The putative full length cDNAs of *NaCYP94B3 like-1*, *-2*, *-3* and *-4* were amplified directly with reverse transcription PCR. NaCYP94B3 like-1, -2 and -4 were relatively similar: the putative protein sequence identities between NaCYP94B3 like-1 and -2, -1 and -4, and -2 and -4 were 97.1, 81.0, and 80.3%, respectively, while the identities between NaCYP94B3 like-3 and NaCYP94B3 like-1, -2, and -4 were only 54.0, 53.7, and 54.0%, respectively (Figure S1). Their protein sequences, together with other AtCYP94B3- and AtCYP94B1-like proteins from some Solanaceous species and all members of the Arabidopsis CYP94 subfamily were phylogenetically analyzed. Compared to the Arabidopsis CYP94B3, the putative protein sequences identities of NaCYP94B3 like-1, -2, -3 and -4 were 60.0, 59.4, 53.0, and 59.3%, respectively; compared to the pepper CaCYP450A, the identities were 87.0 , 87.4, 55.9, and 78.0%, respectively (Figure S1). The four NaCYP94B3 like candidates clustered closely with AtCYP94B1, B2 and B3. Among them, NaCYP94B3 like-1, -2, -3 and -4 are phylogenetically close to AtCYP94B1, B2, and B3, while NaCYP94B3 like-3 is closer to AtCYP94B2 (Figure S1). This result suggests that NaCYP94B3 like-1, -2, -3 and -4 may be the homologues of Arabidopsis CYP94B1, B2 and B3 and hydroxylate JA-Ile.

NaCYP94B3 like-1 **and** ***-2*** **transcripts are strongly induced by wounding and simulated herbivore feeding**

To investigate the role of JA-Ile hydroxylation in plant-herbivore interaction, we first examined the accumulation pattern of 12OH-JA-Ile. *N. attenuata* leaves were treated with mechanical wounding (W+W) or simulated *S. litura* feeding treatment (W+OS). Similar to JA and JA-Ile, 12OH-JA-Ile was transiently induced by W+W: the levels increased quickly and reached the peak level at 2 h; W+OS induced nearly 3-fold more 12OH-JA-Ile than did W+W (Figure 1A), indicating that hydroxylation of JA-Ile is an important process in a plant's response to insect feeding. Additionally, the transient accumulation pattern of 12OH-JA-Ile suggests that it is not a stable JA metabolite and it might be quickly catabolized.

As 12OH-JA-Ile levels are rapidly induced by simulated *S. litura* feeding, we supposed that the genes encoding enzymes involved in JA-Ile hydroxylation are induced by wounding and their expression levels can be amplified by adding OS to wounds. As expected, W+W highly induced *NaCYP94B3 like-1* and *-2* transcripts: *NaCYP94B3 like-1* reached over 2000-fold induction 3 h after the treatment (Figure 1B); *NaCYP94B3 like-2* reached over 1000-fold induction at 1 h after wounding (Figure 1C). W+OS amplified the W+W-induced transcriptional responses: *NaCYP94B3 like-1* and *NaCYP94B3 like-2* expression levels were 2.5 and 1.5 times of those induced by W+W (Figure 1B, 1C), implying that *NaCYP94B3 like-1* and *-2* have functions in plant-herbivore interactions. However, *NaCYP94B3 like-3* and *-4* transcripts were only slightly induced after these treatments: W+W at most induced *NaCYP94B3 like-3* and *-4*, 4- and 2-fold, respectively; W+OS induced *NaCYP94B3 like-3* less than 6-fold and *NaCYP94B3 like-4* only 2.5-fold (Figures 1D and 1E).

Specifically silencing *NaCYP94B3 like-1* and *NaCYP94B3 like-2* reduced the levels of 12OH-JA-Ile

To further examine whether NaCYP94B3 like-1, -2, -3 and -4 hydroxylate JA-Ile in *planta*, gene-specific regions were selected and four VIGS vectors that independently silence the four genes were constructed (Figure S2). The empty vector and the constructed vectors were transformed into *Agrobacterium* to specifically silence these *NaCYP94B3*s using the VIGS technology to generate VIGS-EV, VIGS-NaCYP94B3 like-1, -2, -3 and -4 plants.

As W+OS elicited high transcript levels, we tested the silencing efficiency 1 h after W+OS treatment: the transcripts of the target genes in VIGS-NaCYP94B3 like-1, -2, -3 and -4 plants were 88.2%, 69.4%, 80.1%, and 77.9% silenced, respectively (Figure 2A). We also verified that the four genes were specifically silenced (Figure S3). Notably, no differences in the growth and morphology were found between EV and plants silenced in these genes, even when plants reached the flowering stage (Figure S4). Next, we tested whether silencing the *CYP94B3* homologues would also influence 12OH-JA-Ile and JA-Ile levels after the treatments. One h after W+OS, 12OH-JA-Ile concentrations in VIGS-NaCYP94B3 like-1 and -2 plants were 36.0% and 62.3% decreased, respectively, compared to the concentrations in

VIGS-EV plants; in VIGS-NaCYP94B3 like-3 and -4 plants, 12OH-JA-Ile contents were not significantly lower than those in the VIGS-EV plants (Figure 2B). These results indicate that NaCYP94B3 like-1 and NaCYP94B3 like-2 oxidize JA-Ile to 12OH-JA-Ile. Consistent with the decreased 12OH-JA-Ile levels in plants silenced in *NaCYP94B3 like-1* or *-2*, the JA-Ile levels increased in these plants. As expected, the *NaCYP94B3 like-1*-silenced plants accumulated slightly more (12%) JA-Ile, while *NaCYP94B3 like-2*-silenced plants accumulated substantially more (38%) JA-Ile than did VIGS-EV plants (Figure 2C). JA-Ile is derived from a minor portion of JA, as NaJAR4 and NaJAR6 simultaneously silenced plants does not have elevated JA levels[10]. Consistently, although specifically silencing *NaCYP94B3 like-1* and *-2* increased the levels of JA-Ile, the levels of JA were not affected (Figure S5). These results suggested that impairing JA-Ile hydroxylation increases JA-Ile, but not JA, accumulations in plants.

Simultaneously silencing *NaCYP94B3 like-1*, *-2* and *-4* enhances *N. attenuata*'s resistance to the insect *S. litura*

As silencing *NaCYP94B3 like-1* and *-2* both decreased the levels of hydroxylated JA-Ile, we speculated that these genes may act redundantly. A VIGS vector with a region that targeted the transcripts of *NaCYP94B3 like-1*, *-2* and *-4* (as *NaCYP94B3 like-4* shares high sequence similarity to *NaCYP94B3 like-1* and *-2*), but not *NaCYP94B3 like-3*, was constructed and transformed into *Agrobacterium* to generate silenced plants; the resulted plants were called VIGS-NaCYP94B3s (Figure S2). Again, no differences in the growth and morphology were found between VIGS-EV and VIGS-NaCYP94B3s plants (Figure S6). One h after W+OS treatment, *NaCYP94B3 like-1*, -2, *-4* were 56.8%, 62.3%, and 43.9% silenced in VIGS-NaCYP94B3s plants, respectively (Figure 3A). Importantly, VIGS-NaCYP94B3s plants accumulated only 18.0% of the 12OH-JA-Ile levels detected in VIGS-EV plants (Figure 3B), and JA-Ile levels in NaCYP94B3s plants were over 2 times that of those in VIGS-EV plants (Figure 3C), indicating the NaCYP94B3 like-1- and -2-mediated hydroxylation is a major turnover route of JA-Ile. Given that specifically silencing *NaCYP94B3 like-4* did not affect the levels of 12OH-JA-Ile, the impaired 12OH-JA-Ile levels in simultaneously silenced plant mainly resulted from the silencing of *NaCYP94B3 like-1* and *-2*. Additionally, similar to the results of silencing the NaCYP94B3 homologues, the simultaneous silencing the three *NaCYP94B3 like* homologues also did not affect JA levels (Figure 3D).

To further examine the role of JA-Ile hydroxylation in plant-herbivore interaction, we compared the larval masses of *S. litura* growing on VIGS-NaCYP94B3s and VIGS-EV plants. Eleven days after feeding on VIGS-NaCYP94B3s plants, the larvae were significantly smaller than those grown on VIGS-EV plants. After 15 days, the differences were more obvious: the average mass of the larvae on VIGS-NaCYP94B3s plants was only half of the average insect mass on VIGS-EV plants (Figure 4A). This result indicates that 12OH-JA-Ile does not activate plant defenses against this herbivore. We reasoned that the significantly decreased larval mass on VIGS-NaCYP94B3s plants resulted from the elevated JA-Ile levels. To test this, we use the same construct to silence NaCYP94B3 homologues in *irjar4/6* plants, which have highly decreased JA-Ile levels, and compared *S. litura* larval masses. When we silencing NaCYP94B3 homologues in the WT background, 14 days after feeding on VIGS-NaCYP94B3s plants, the larvae were significantly smaller than those grown on

VIGS-EV plants. In contrast, when we used the same construct in the *irjar4/6* background, the larvae grown on these VIGS-NaCYP94B3s plants did not differ from those grown on VIGS-EV plants (Figure S7A). Meanwhile, in the WT background, the JA-Ile and 12OH-JA-Ile levels of VIGS-NaCYP94B3s plants were over 1.5-fold higher and over 1-fold lower than those of VIGS-EV plants, respectively. In the *irjar4/6* background, the levels of JA-Ile and 12OH-JA-Ile were not different between VIGS-EV and VIGS-NaCYP94B3s plants (Figure S7B, C). These results indicate that the increased *S. litura* resistance in *NaCYP94B3s*-silenced plants was caused by the elevated JA-Ile levels.

NaCYP94B3 homologues attenuates multiple herbivore-induced direct defenses

JA-Ile induces plants accumulating a number of secondary metabolites that directly suppress the performance of caterpillars[1, 10, 34]. To investigate whether the over-accumulation of JA-Ile caused by blocking JA-Ile hydroxylation induces defense-related secondary metabolites, we measured several direct defense traits in VIGS-NaCYP94B3s plants. As we expected, the three main direct defense traits, namely, TPI activity, nicotine contents, and HGL-DTGs levels, were all highly induced in VIGS-NaCYP94B3s plants. Three days after W+OS treatment, the TPI activity, total HGL-DTGs and nicotine levels in VIGS-NaCYP94B3s were 57.8%, 32.3%, and 50.1% (Figure 4B-D) higher than those in the VIGS-EV plants. Over-accumulation of these metabolites in VIGS-NaCYP94B3s plants accounted for the reduced performance of caterpillars on VIGS-NaCYP94B3s plants.

Accumulation of 12OH-JA-Ile requires JA-Ile and COI1

To gain insight into how herbivory-induced 12OH-JA-Ile accumulation is regulated, we examined the JA, JA-Ile and 12OH-JA-Ile accumulation patterns in WT, *iraoc*, *irjar4/6* and *ircoi1* plants, which have impaired JA biosynthesis[27], JA-Ile biosynthesis[10], and JA perception[28], respectively, after W+OS treatment. The JA levels in the four genotypes were very different: At 1 h, WT plants showed highly increased JA contents after W+OS, while JA levels in *iraoc* plants were strongly reduced to < 3% of the WT levels as the *iraoc* plants were impaired in JA production; the JA levels in *irjar4/6* plants were similar to those in WT plants, and consistent with the previous report, JA level in *ircoi1* plants was ~30% of those in WT plants [35] (Figure 5A). In the case of JA-Ile: in WT plants, JA-Ile retained the transient accumulation pattern that followed that of JA; at 1 h, JA-Ile levels in *iraoc* plants were also strongly reduced to < 3% of WT levels and *irjar4/6* plants had only 30% of the JA-Ile contents in WT plants; remarkably, although JA levels were very low in *ircoi1* plants, the JA-Ile levels in *ircoi1* plants increased to levels found in WT plants and the levels remained high until 3 h when JA-Ile levels had returned to basal levels in WT plants, suggesting the JA-Ile turnover pathway was somewhat blocked in *ircoi1* plants (Figure 5B). 12OH-JA-Ile also transiently accumulated like JA and JA-Ile in WT plants, and compared with its levels in WT plants, *iraoc* (< 1% at 1 h), *irjar4/6* (< 14% at 1 h), and *ircoi1* (< 16% at 1 h) all exhibited strongly decreased 12OH-JA-Ile contents (Figure 5C). Thus, the production of 12OH-JA-Ile requires an intact JA-Ile biosynthesis and perception pathway.

We inferred that the reduced 12OH-JA-Ile levels in *irjar4/6* and *iraoc* plants resulted from substrate limitations. To test this possibility, we exogenously applied JA and JA-Ile to WT, *iraoc*, *irjar4/6* and *ircoi1* plants and measured 12OH-JA-Ile levels. When JA was supplied to the wounds of the plants with OS (W+OS+JA), WT plants did not accumulate more 12OH-JA-Ile than did W+OS, and similarly, exogenous JA supplementation did not recover the accumulation of 12OH-JA-Ile in *irjar4/6* and *ircoi1* plants, even though *ircoi1* plants had XXX times more JA-Ile than did WT plants (Figure 5D); JA only partly rescued the 12OH-JA-Ile levels in *iraoc* plant, as the JA-Ile levels in *iraoc* plant were only half of those in WT plant (Figure 5E). In contrast, exogenous treatments of JA-Ile increased the 12OH-JA-Ile levels (> 7 fold) in WT plant (Figure 5D). Importantly, JA-Ile also completely recovered the 12OH-JA-Ile levels in *iraoc* and *irjar4/6* plants. However, JA-Ile could not rescue the 12OH-JA-Ile levels in *ircoi1* plant (Figure 5D). Notably, exogenous application of JA did not enhance JA-Ile levels in WT but elevated JA-Ile contents *iraoc* plants (Figure 6B), and this was likely due to possibility that 1) the high levels of JA in these plants (induction by W+OS treatment and supplementation of JA) saturated the mechanism of JA-Ile production; 2) the highly increased JA-Ile levels in *ircoi1* plants (Figure 6B) were resulted from the decreased activity of CYP94B3s in these plants.

These results indicate that the biosynthesis of 12OH-JA-Ile is JA-Ile as well as COI1-dependent.

COI1 regulates JA-Ile hydroxylation by controlling the transcript levels of *NaCYP94B3 like-1* and *NaCYP94B3 like-2*

To further investigate whether 12OH-JA-Ile synthesis is transcriptionally regulated by COI1, we investigated the expression patterns of *NaCYP94B3 like-1* and *-2* in *ircoi1* plants, and found that the transcript levels of *NaCYP94B3 like-1* and *-2* were greatly decreased in *ircoi1* (Figure 6): At 1h after W+OS treatment, *NaCYP94B3 like-1* transcript levels were less than 20% of those in WT plants, and in addition the peak at 3 h was gone. Meanwhile, *NaCYP94B3 like-2* also accumulated only 12.1% of the transcript abundance of WT plant 1 h after W+OS treatment. These results indicate that JA signaling regulates 12OH-JA-Ile synthesis by activating the transcription of *NaCYP94B3-like-1* and *-2*.

A previous study indicated that COI1 positively regulates both jasmonate biosynthesis and JA-Ile metabolism [35]. Given that the hydrolysis of JA-Ile back to JA is another catabolic route of JA-Ile, we further investigated whether COI1 also regulates the transcription of *NaJIH1* (*jasmonoyl-L-isoleucine hydrolase 1*), the JA-Ile hydrolase [19]. W+OS induced the transcripts of *NaJIH1* rapidly. At 2 h after W+OS, the level of *NaJIH1* was over 25-fold of that in untreated WT plants, whereas in *ircoi1* plants, W+OS only induced a maximal 5 fold increase of *NaJIH1* transcripts (Figure S8). Thus, JA signaling also regulates the transcript abundance of JA-Ile hydrolase NaJIH1. Taken together, the impairments of two main JA-Ile catabolic routes likely accounts for JA-Ile over-accumulation phenotype of *ircoi1* plants.

DISCUSSION

Plants are often challenged by various inset herbivores. In plant defense against insects, the phytohormone JA and JA-Ile play important roles [1, 10, 11]. Here we show that NaCYP94B3s are central in catabolizing JA-Ile, thus is an important regulator in maintaining JA-Ile homeostasis.

NaCYP94B3s are important for the homeostasis of the herbivory-induced defense signal molecule, JA-Ile

In response to herbivore attack, plant quickly synthesis the signal molecule, JA-Ile, which elicits induce defenses[11]. However, prolonged or over- accumulation of JA-Ile is unnecessary and even causes growth retardation [16]. Oxidation and conjugation with sugar are common inactivation routes for phytohormones, and oxidation is mainly carried out by P450s[36].

N. attenuata employs CYP94B3 homologues to tailor the levels of herbivore-induced signal, JA-Ile. Four genes in *N. attenuata* (*NaCYP94B3 like-1*, *2*, *3* and *4*) were found to have high homologies with Arabidopsis *CYP94B3s*. In response to simulated *S. litura* attack, *NaCYP94B3 like-1* and *like-2* were strongly induced, while *NaCYP94B3 like-3* and *-4* was only slightly or not induced. Independently silencing the four genes further indicates both NaCYP94B3 like-1 and -2 hydroxylate JA-Ile. Simultaneously silencing *NaCYP94B3 like-1* and *-2* reduced the levels of 12OH-JA-Ile to less than 20% of those in control plants. Thus, NaCYP94B3 like-1 and -2 govern the majority of JA-Ile hydroxylation. Simultaneously silenced plants accumulated 1 fold more JA-Ile than did control plants; this clearly indicates that 12-hydroxidation by these P450s is the main turnover route of JA-Ile. A similar mechanism has been identified in Arabidopsis: double mutant of *CYP94B1* and *CYP94B3* lost the ability of producing 12OH-JA-Ile, and the expression of two *CYP*s, were *coi1*-dependent[20, 21]. It is possible that in eudicots, hydroxylation of JA-Ile is usually carried out by an evolutionarily conserved small CYP gene family.

In *N. attenuata*, another important turnover route of JA-Ile is hydrolyzing JA-Ile back to JA by jasmonoyl-L-isoleucine hydrolase 1 (NaJIH1). *NaJIH1*-silenced plants accumulated about 2-fold higher JA-Ile and were also more resistant to herbivores [19]. Whether there are any other JA-Ile catabolic pathways would be very interesting to explore.

In response to simulated herbivory, plants synthesize large amount of JA: the peak level of JA is about 1.2 μg g^{-1} FW. JA is quickly catabolized: 2 h after W+OS elicitation, its levels are almost back to basal levels (Figure 5A). Notably, JA-Ile is a minor form of JA metabolites. Only about 1/3 of the JA pool is converted to JA-Ile, as the peak level of JA-Ile is about 0.4 μg g-1 FW. It is likely that most of the elicited JA is metabolized to compounds other than JA-Ile, such as hydroxylated jasmonates 12OH-JA, 12-HSO4-JA and 12-O-Glc-JA, which are constituents of various organs of many plant species. In tomato's response to wounding, these compounds all accumulate differentially and usually to much higher concentrations than does JA[37]. Although we did not measure the profiles of these hydroxylated jasmonates after simulated herbivore responses in *N. attenuata*, we speculate they are also main metabolites of JA. Whether these metabolites have any functions in plant-herbivore interaction remains to be explored.

JA signaling regulates the metabolism of JA-Ile

The F-box protein COI1 is involved in jasmonate perception[8, 9, 38, 39]. In addition, COI1 also has regulatory activity on oxylipin biosynthesis: In Arabidopsis, the transcripts of JA-Ile biosynthesis enzymes are controlled by COI1[40] and NaCOI1 is required for the transcriptional induction of JA biosynthesis genes, including *NaAOS*, *NaAOC*, *NaOPR3*, *NaJAR4* and *NaACX1* in *N. attenuata*[35]. It was speculated previously that COI1 also regulates the metabolism of JA-Ile[35]. In *NaCOI1* knock-down plants (*ircoi1*), JA-Ile levels were higher and the elicited peak more prolonged than in WT plants, while the levels of JA were much lower [35]. *In vivo* substrate feeding experiments in *N. attenuata* further demonstrated that JA-Ile accumulation pattern in *ircoi1* is not the result of altered substrate availability, i.e. of JA and/or Ile, but is due to an over 6-fold decrease in JA-Ile turnover [35].

In this study, we found that JA signaling regulates the turnover of JA-Ile by activating the transcription of JA-Ile catabolic enzymes. Both hydroxylation (*NaCYP94B3 like-1* and *-2*) and hydrolysis (*NaJIH1*) enzymes are transcriptionally controlled by JA signaling, given that they all had very low level of transcripts in *ircoi1* plants. Clearly, the low metabolic rate of JA-Ile is the reason for the increased and prolonged accumulation of JA-Ile in *ircoi1* plants. The results in *N. attenuata* further indicate that after herbivore feeding, JA signaling fine-tunes the dynamics of the jasmonate response by regulating the dynamics of the transcription of JA biosynthesis and catabolism enzymes. Considering COI1 is an F-box protein, it may not regulate the transcription of jasmonate biosynthesis and metabolism genes directly. Possibly, the COI1 downstream transcription factor, MYC2, is the direct regulator of these genes.

CYP94B3 homologues are potential targets for genetic engineering of herbivore-resistant crops

Silencing the transcripts of *NaCYP94B3 like-1* and *-2* resulted in highly resistant *N. attenuata* plants to the generalist herbivore *S. litura*. Consistently, these plants produced more defense-related metabolites: nicotine, HGL-DTGs, and TPIs, and these were correlated with the doubled JA-Ile levels. Although we did not test the susceptibility of the silenced plant to other insects, such as the specialist insect *M. sexta*, elevated defense levels are expected to generally increase resistance to many insect herbivore species[41]. So far, most identified genes involved in defenses responses against insect herbivore play positive roles; in contrast, our results indicate that silencing or knocking out *CYP94B3*s enhances plant resistance to insect herbivores. Importantly, under our cultivation conditions, we did not detect abnormal growth of the plants silenced in *NaCYP94B3*s. We speculate that either residual levels of these enzymes' activity or other non-silenced *NaCYP94B3* homologues as well as NaJIH1 were sufficient to maintain JA/JA-Ile homeostasis under normal conditions. This is essential, as constitutively elevated JA and/or JA-Ile are known to suppress plant growth.

We propose that CYP94B3 homologues have potential values in generating herbivore-resistant plants by breeding or transgene methods. CRISPR/Cas9 and other genome editing technologies have started to become feasible for crop plants[42-44], which usually generate gene knock-outs. Thus, using these new genome-editing technologies, knocking out the expression of *CYP94B3*s in crops could be done to examine whether these P450s could serve as new targets of crop improvement.

SUPPORTING INFORMATION

Phylogenetical analysis of CYP94 proteins **(**Figure S1), a multiple sequence alignment of the four NaCYP94B3 likes **(**Figure S2), independently silencing NaCYP94B3 likes by VIGS did not change JA levels (Figure S3), specifically silencing NaCYP94B3 likes by VIGS does not change plant morphology (Figure S4), independent silencing *NaCYP94B3 like* homologues does not change JA levels (Figure S5), simultaneously silencing NaCYP94B3 likes by VIGS did not change plant morphology (Figure S6), simultaneously silencing NaCYP94B3 homologues increased *S. litura* resistance is caused by elevated JA-Ile levels (Figure S7), COI1 regulates the transcript levels of *NaJIH1* (Figure S8), and list of primers (Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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NOTES

The authors declare no competing financial interest.

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FIGURE CAPTIONS

Figure 1. Profiles of 12OH-JA-Ile, JA and JA-Ile contents in *N. attenuata* plants and transcript levels of *NaCYP94B3-like* genes after W+W and W+OS elicitation.

Leaves were wounded with a fabric pattern wheel, and the resulting puncture wounds (W) were immediately treated with 20µL of double-distilled H_2O (W+W) (dashed line, closed diamonds) or 1:5-diluted *S. litura* oral secretions and regurgitants (W + OS) (solid lines, closed squares). Leaf tissues were harvested after indicated times. Contents of jasmonates 12-hydroxy-jasmonyl-isoleucine (12OH-JA-Ile), jasmonic acid (JA) and jasmonyl-isoleucine (JA-Ile) were determined using HPLC-MS/MS (A). Transcripts levels of *NaCYP94B3 like-1* (B), *NaCYP94B3 like-2* (C), *NaCYP94B3 like-3* (D), and *NaCYP94B3 like-4* (E) were quantified with qPCR. (n =3 to 5; error bars represent ± SE).

Figure 2. Specifically silencing of *NaCYP94B3 like*s by VIGS reduces 12OH-JA-Ile accumulations.

Plants were inoculated with *Agrobacterium* harboring TRV constructs to obtain VIGS-EV, VIGS-NaCYP94B3 like-1, VIGS-NaCYP94B3 like-2, VIGS-NaCYP94B3 like-3 and VIGS-NaCYP94B3 like-4 plants. Leaves were wounded with a pattern wheel and puncture wounds were immediately treated with 1:5-diluted *S. litura* oral secretions (OS), and leaf tissues were harvested after 60 min. Silencing efficiency of *NaCYP94B3 like*s (A), contents of 12OH-JA-Ile (B), and JA-Ile concentrations (C) in these plants. Experiments were performed with 4 to 5 biological replicates; error bars represent ± SE. *Asterisks* represent significant difference between members of a pair (student's t-test: **, $P < 0.01$).

Figure 3. Simultaneously silencing *NaCYP94B3 like*s by VIGS reduces 12OH-JA-Ile accumulation, but increases JA-Ile levels.

Plants were inoculated with *Agrobacterium* harboring TRV constructs to obtain VIGS-EV and VIGS-NaCYP94B3s plants. Leaves were wounded with a pattern wheel and the resulting puncture wounds immediately applied with 1:5-diluted *S. litura* oral secretions (OS), and leaf tissues were harvested after 60 min. (A) Transcripts of *NaCYP94B3 like-1*, *NaCYP94B3 like-2*, and *NaCYP94B3 like-4*, but not *NaCYP94B3 like-3*, were silenced in VIGS-NaCYP94B3s plants. Relative concentrations of 12OH-JA-Ile (B), and contents of JA-Ile (C) and JA (D) in VIGS-EV and VIGS-NaCYP94B3s plants. (n = 4 to 5; error bars represent ± SE; *Asterisks* represent significant differences between members of a pair (Student's t test: *, $P< 0.05$; ***, $P< 0.001$; ****, $P< 0.0001$).

Figure 4. Plant resistance to *S. litura* and direct defenses are increased in plants silenced in *NaCYP94B3 like* expression.

(A) *S. litura* larval mass on VIGS-EV and VIGS-NaCYP94B3s plants. Two freshly hatched larvae were placed on leaves of VIGS-EV and VIGS-NaCYP94B3s plants. For each group, 18-20 larvae were used. TPI activity (B), nicotine levels (C), and total HGL-DTGs (D) in VIGS-EV and VIGS-NaCYP94B3s plants. VIGS-EV and VIGS- NaCYP94B3s plants were wounded, and treated with 1: 5-diluted *S. litura* OS. Leaf tissues were harvested after 72 h for direct defenses analysis. (n = 4 to 5; error bars represent ± SE. Asterisks represent significant differences between different plants (Student's t test: *, $P< 0.05$; **, $P< 0.01$; ***, $P< 0.001$).

Figure 5. Accumulation of 12OH-JA-Ile require JA-Ile and COI1

Jasmonate profiles of *N. attenuata* WT, *iraoc*, *irjar4/6* and *ircoi1* plants .WT, *iraoc*, *irjar4/6* and *ircoi1* plants were elicited with W+OS, and leaves were harvested after different times. JA (A), JA-Ile (B), and 12-OH-JA-Ile (C). The accumulation of 12OH-JA-Ile is dependent on JA-Ile and *NaCOI1*.12OH-JA-Ile (D) and JA-Ile levels (E) in WT, *iraoc*, *irjar4/6* and *ircoi1* plants 1 h after puncture wounds were treated with W+OS, W+OS+JA or W+OS+JA-Ile. Contents were determined with HPLC-MS/MS. (n = 3 to 5; error bars represent ± SE).

Figure 6. Transcript levels of *NaCYP94B3-like-1* and *-2* are dependent on *NaCOI1*.

NaCYP94B3 like-1 (A) and *-2* (B) transcripts levels in WT and *ircoi1* plants after W + OS elicitation. Plants were W+OS elicited, and leaf tissue was harvested after indicated times. Total RNA was isolated and transcript levels were quantified by qPCR. (n = 3 to 5; error bars represent ± SE).

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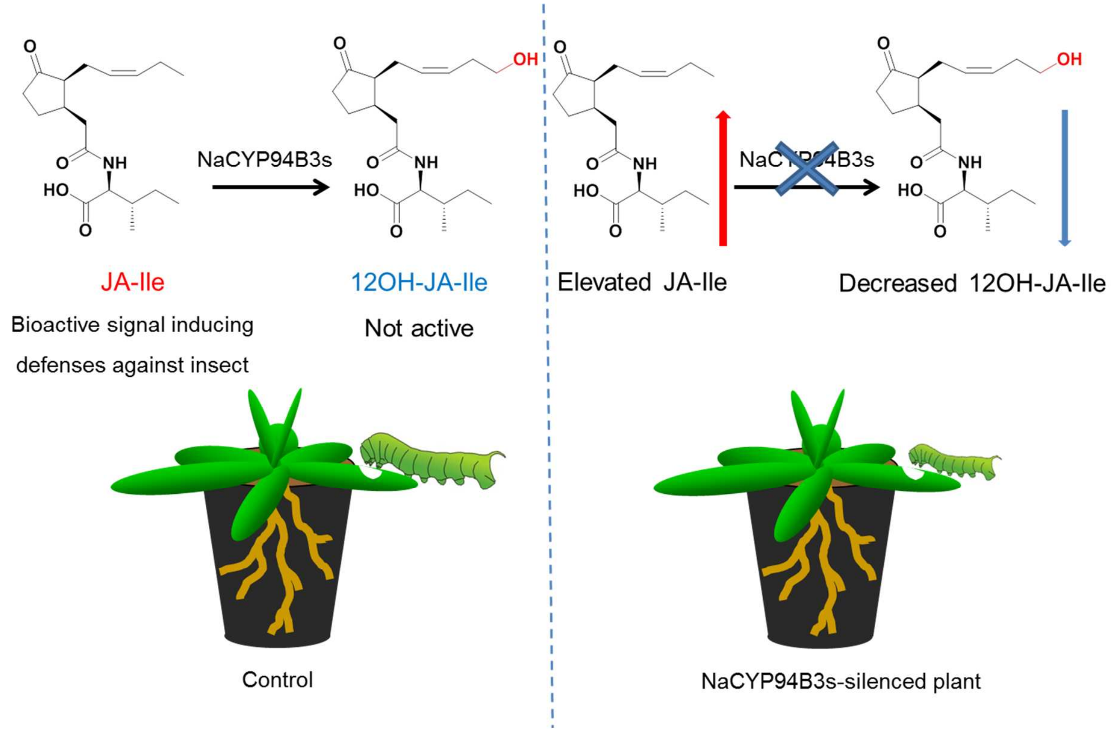

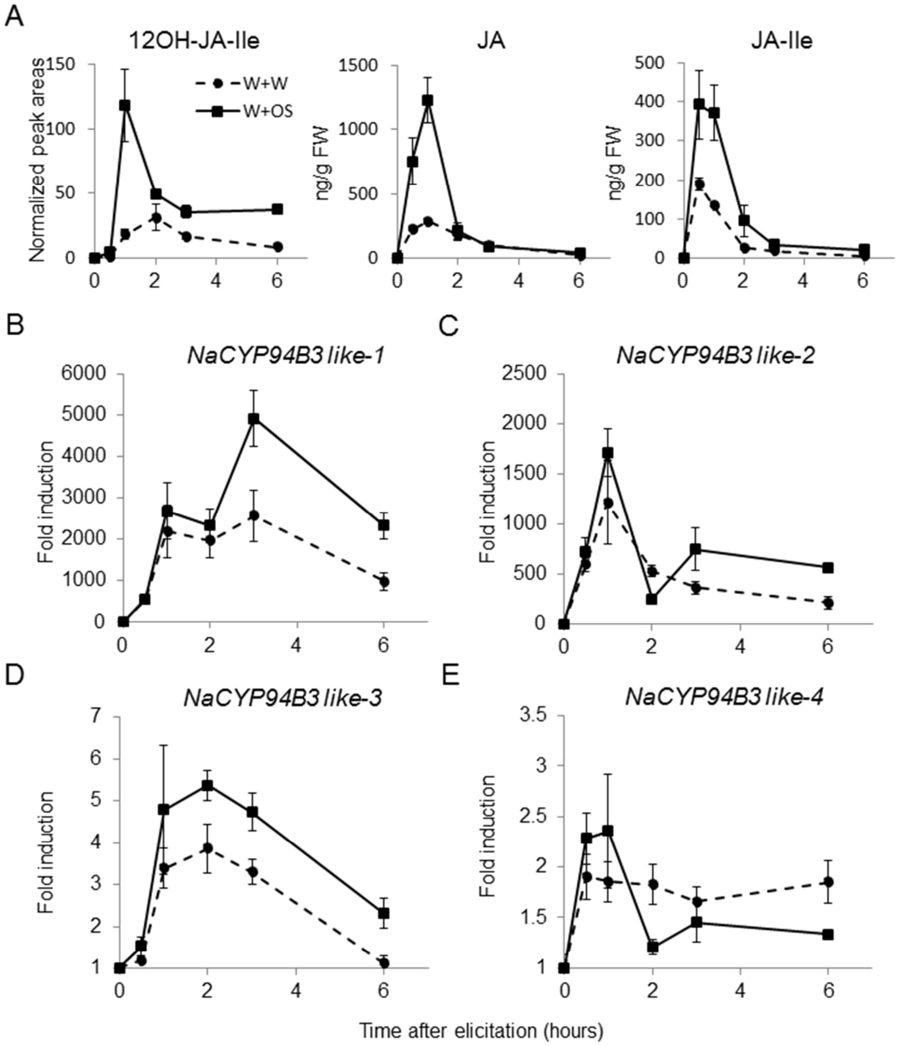

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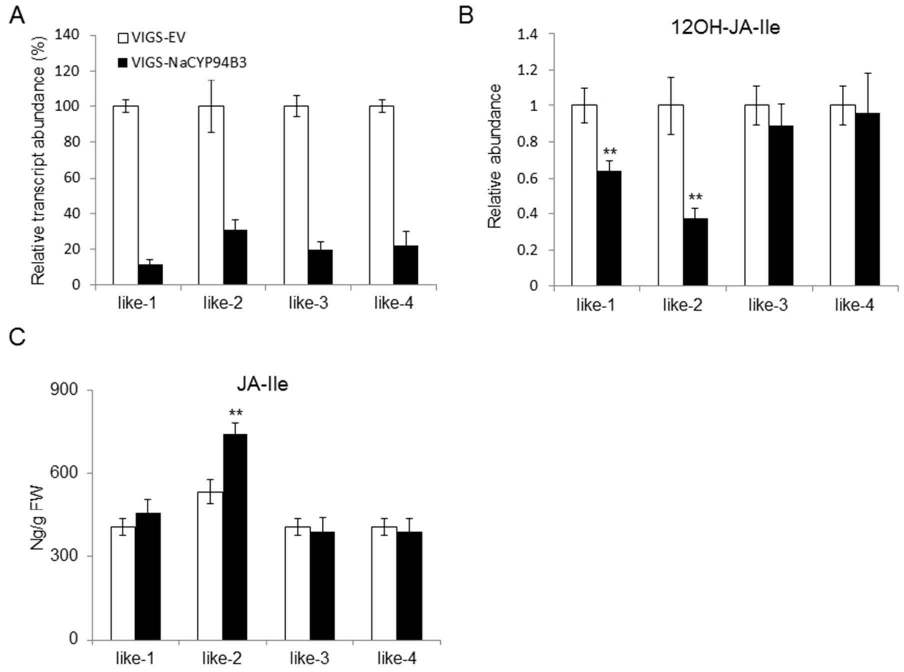

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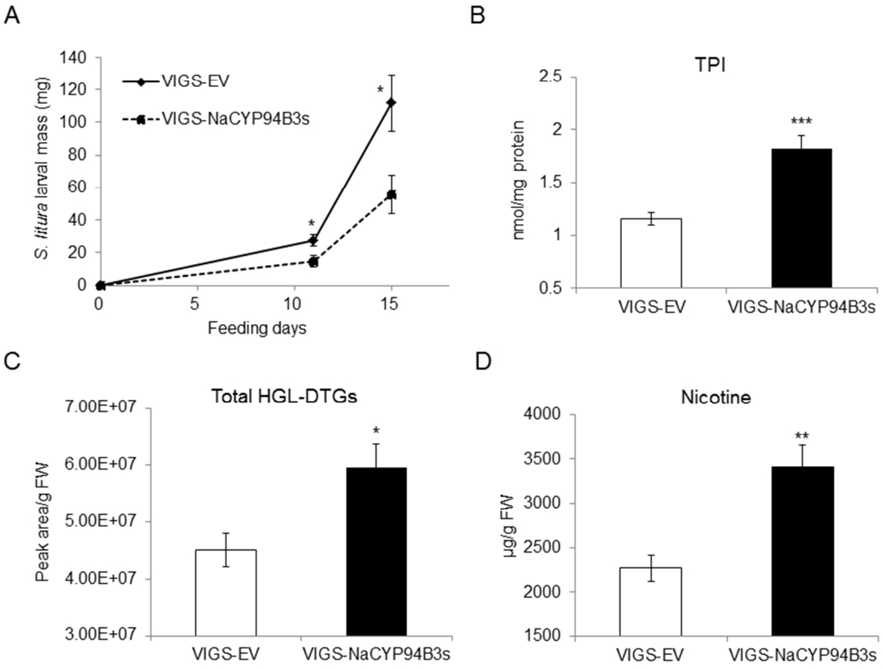

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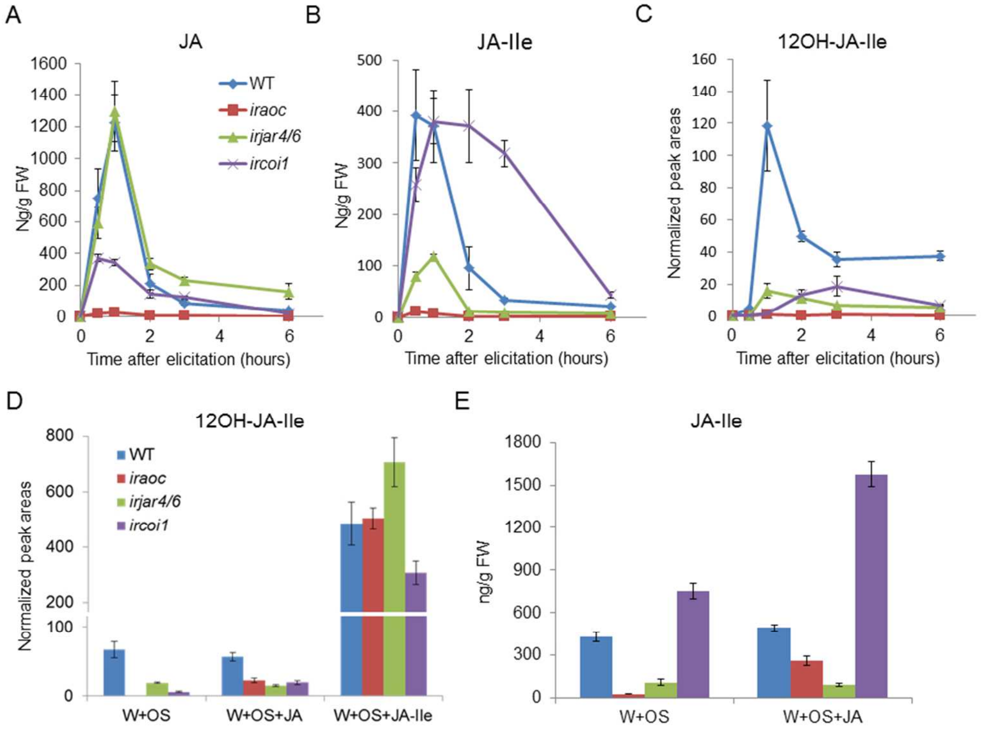

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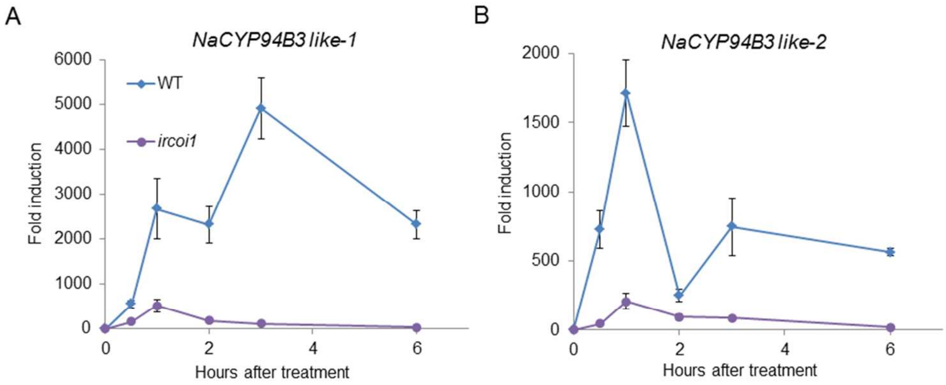

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