Cytochrome P450 CYP94B3 mediates catabolism and inactivation of the plant hormone jasmonoyl-L-isoleucine

Abraham J. K. Koo[a,b], Thomas F. Cooke[a,b,1], and Gregg A. Howe[a,b,2]

[a]Department of Energy-Plant Research Laboratory and [b]Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI 48824

Edited by Mark Estelle, University of California at San Diego, La Jolla, CA, and approved April 27, 2011 (received for review March 3, 2011)

The phytohormone jasmonoyl-L-isoleucine (JA-Ile) signals through the COI1-JAZ coreceptor complex to control key aspects of plant growth, development, and immune function. Despite detailed knowledge of the JA-Ile biosynthetic pathway, little is known about the genetic basis of JA-Ile catabolism and inactivation. Here, we report the identification of a wound- and jasmonate-responsive gene from *Arabidopsis* that encodes a cytochrome P450 (CYP94B3) involved in JA-Ile turnover. Metabolite analysis of wounded leaves showed that loss of CYP94B3 function in *cyp94b3* mutants causes hyperaccumulation of JA-Ile and concomitant reduction in 12-hydroxy-JA-Ile (12OH-JA-Ile) content, whereas overexpression of this enzyme results in severe depletion of JA-Ile and corresponding changes in 12OH-JA-Ile levels. In vitro studies showed that heterologously expressed CYP94B3 converts JA-Ile to 12OH-JA-Ile, and that 12OH-JA-Ile is less effective than JA-Ile in promoting the formation of COI1-JAZ receptor complexes. CYP94B3-overexpressing plants displayed phenotypes indicative of JA-Ile deficiency, including defects in male fertility, resistance to jasmonate-induced growth inhibition, and susceptibility to insect attack. Increased accumulation of JA-Ile in wounded *cyp94b3* leaves was associated with enhanced expression of jasmonate-responsive genes. These results demonstrate that CYP94B3 exerts negative feedback control on JA-Ile levels and performs a key role in attenuation of jasmonate responses.

plant defense | jasmonate receptor | jasmonic acid | plant–insect interaction | fatty acid hydroxylase

The fatty acid-derived hormone jasmonate plays a central role in regulating plant growth, reproduction, and responses to biotic stress. A wealth of genetic and biochemical evidence indicates that jasmonoyl-L-isoleucine (JA-Ile) is a receptor-active form of the hormone (1–4). This conclusion is supported by structural studies showing that the jasmonic acid (JA) and isoleucine moieties of JA-Ile serve critical roles in the assembly of an intracellular COI1-JAZ receptor complex in which JAZ repressors are targeted for ubiquitination by the E3 ligase SCF^{COI1} (5). Degradation of JAZ repressors by the 26S proteasome activates genome-wide transcription of genes involved in various jasmonate responses (1–4). The central role of JA-Ile in initiating this signaling pathway highlights the importance of understanding cellular processes involved in JA-Ile homeostasis.

JA-Ile is well characterized for its function in orchestrating plant defense responses to wounding and insect attack (6, 7). In the *Arabidopsis* rosette, mechanical tissue damage triggers rapid local and systemic increases in JA-Ile levels, degradation of JAZ repressors, and activation of gene expression (8–12). The transient nature of wound-induced JA-Ile accumulation implies the existence of mechanisms to inactivate or otherwise remove JA-Ile from stimulated cells. Among the pathways implicated in catabolism of the hormone are conversion of JA and JA-Ile to their corresponding 12-hydroxy derivatives (12OH-JAs), 12OH-JA (also known as tuberonic acid) and 12OH-JA-Ile, respectively (11, 13–15). The 12OH-JAs can be further metabolized to sulfo- and glucosyl derivatives that are largely inactive in promoting responses typically attributed to jasmonate (13, 16, 17). However, the ability 12OH-JAs to induce certain physiological responses, including tuber formation and leaf closing, raises the possibility that these compounds signal independently of the COI1-JAZ receptor system (17, 18).

Despite the important biological properties and widespread occurrence of 12OH-JAs in the plant kingdom, enzymes responsible for 12-hydroxylation of JA and JA-Ile have not been reported. Here, we identify JA-Ile-12-hydroxylase as a member (CYP94B3) of the CYP94 family of cytochrome P450 monooxygenases (P450s). Functional studies using genetic, biochemical, and metabolic approaches demonstrate a role for CYP94B3 in JA-Ile turnover and attenuation of jasmonate responses. These findings thus reveal a previously unexplored class of enzymes that perform a key role in jasmonate metabolism and signaling.

Results

***CYP94B3* Encodes a JA-Ile-12-Hydroxylase.** We used two general criteria to identify candidate genes encoding JA-Ile-12-hydroxylase. First, the prominent role of P450s in small-molecule hydroxylation and phytohormone inactivation suggested their potential involvement in the synthesis of 12OH-JAs. Because 12OH-JAs are hydroxylated at the ω position of the fatty acyl-derived JA moiety (Fig. 1*A*), we focused our attention on members of the CYP86 and CYP94 families of P450 that are well characterized for their role in fatty acid ω-hydroxylation (Fig. 1*B*) (19–21). Second, based on the kinetics of substrate (JA/JA-Ile) and product (12OH-JAs) accumulation during the wound response (11, 13), we reasoned that genes encoding enzymes involved in JA-Ile turnover may be induced by wounding. Mining of publically available gene expression data (22) enabled us to refine the list of candidates to three *CYP94* genes whose expression is strongly induced by wounding and JA treatment: *CYP94B1* (At5g63450), *CYP94B3* (At3g48520), and *CYP94C1* (At2g27690). RNA blot experiments confirmed that the expression of all three genes is wound-inducible, coregulated with the JA biosynthetic gene *OPR3*, and dependent on COI1 (Fig. 1*C*). Consistent with previous studies showing that the COI1 pathway promotes JA-Ile turnover (23), we found that wounded leaves of the *coi1-1* mutant accumulate higher levels of JA-Ile and lower levels of 12OH-JA-Ile than leaves of WT plants (Fig. S1).

To test whether the selected CYP94s affect JA-Ile-12-hydroxylase activity *in planta*, we determined the pattern of wound-induced

Author contributions: A.J.K., and G.A.H. designed research; A.J.K. and T.F.C. performed research; A.J.K. and G.A.H. analyzed data; and A.J.K. and G.A.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]Present address: Biology Department, Stanford University, Stanford, CA 94305-5020.

[2]To whom correspondence should be addressed. E-mail: howeg@msu.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1103542108/-/DCSupplemental.

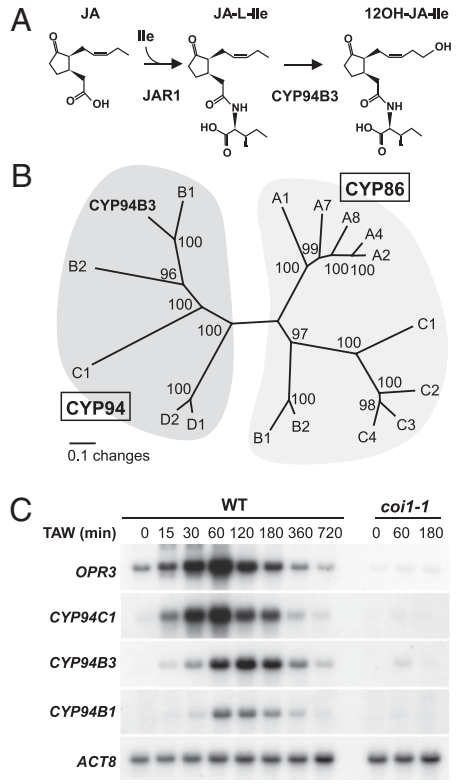

Fig. 1. Identification of candidate cytochrome P450s involved in 12-hydroxylation of JA-Ile. (*A*) Schematic of reactions catalyzed by JAR1 and JA-Ile-12-hydroxylase (CYP94B3). (*B*) Unrooted neighbor-joining phylogeny of the CYP86 and CYP94 subfamily of P450s in *Arabidopsis*, including bootstrap values for each branch. (*C*) RNA blot analysis showing the wound-induced expression pattern of three candidate *CYP94* genes and a JA biosynthesis gene (*OPR3*) in WT and *coi1-1* mutant plants. *ACT8* was included as loading control. TAW, time after wounding.

accumulation of JA-Ile and 12OH-JA-Ile in leaves of T-DNA insertion mutants that fail to express *CYP94* transcripts (Fig. S2). In WT plants, JA-Ile levels rose rapidly within 30 min of wounding, peaked at 1 h, and gradually declined at later time points (Fig. 2*A*). After a lag period of ~30 min 12OH-JA-Ile accumulated and increased steadily for the remainder of the time course (Fig. 2*A*). The JA-Ile and 12OH-JA-Ile content in *cyp94b1* and *cyp94c1* lines was not significantly different from that of WT plants (Fig. S3). In contrast, the amount of JA-Ile produced in wounded *cyp94b3-1* leaves was three- to four-times that in WT leaves. This massive increase in JA-Ile was accompanied by a large decrease (<10% WT levels) in 12OH-JA-Ile levels (Fig. 2 *A* and *B*). Similar results were obtained with a line harboring an independent T-DNA insertion (*cyp94b3-2*) in *CYP94B3* (Fig. S3).

We used additional mutants to further test the role of CYP94B3 in JA-Ile catabolism. Wounded leaves of transgenic lines (*CYP94B3-OE*) that overexpress CYP94B3 were severely depleted in JA-Ile (Fig. 2 *A* and *B*). The 12OH-JA-Ile content in these plants was elevated at early time points (30 min) after wounding but, at later (4 h) time points, was reduced in comparison with WT. Similar to *cyp94b3* mutants, wounded leaves of the *jar1* mutant that is defective in JA conjugation to Ile contained very low levels of 12OH-JA-Ile. However, in contrast to the elevated JA-Ile content in *cyp94b3* plants, wounded *jar1* leaves produced very low levels of JA-Ile (Fig. 2*B*). These findings are consistent with a pathway in which wound-induced production of 12OH-JA-Ile depends on the concerted action of JAR1 and CYP94B3 (Fig. 1*A*).

We expressed CYP94B3 in yeast (*Saccharomyces cerevisiae*) to directly test its function as a JA-Ile-12-hydroxylase. Microsomal fractions prepared from yeast cells transformed with either the empty vector (pYeDP60) or with the *CYP94B3* ORF were incubated with JA-Ile, and the reaction products were analyzed by LC-MS/MS. Microsomes from *CYP94B3*-expressing cells produced low but detectable amounts of 12OH-JA-Ile (Fig. 2*C*). In reactions containing microsomes from empty vector-containing control cells 12OH-JA-Ile was not produced, thus demonstrating that CYP94B3 has JA-Ile-12-hydroxylase activity.

Ectopic Expression of CYP94B3 Recapitulates JA-Deficient Phenotypes. We observed abnormal silique development and reduced seed set in 4 of 25 independent *CYP94B3-OE* T1 lines selected for the presence of the transgene (Table S1). This defect in fertility was tightly correlated with reduced JA-Ile levels and increased 12OH-JA-Ile content in wounded leaves of plants within the T1 population (Fig. S4), and was also heritable in subsequent generations (Fig. 3*A*). Developing flowers from affected *CYP94B3-OE* lines exhibited extended stigma papillae, short anther filaments, and reduced pollen viability (Fig. 3 *B* and *C*). These reproductive phenotypes are typical of *Arabidopsis* mutants that are defective in JA synthesis or perception (2).

We next used homozygous *CYP94B3-OE* lines to determine whether overexpression of CYP94B3 affects jasmonate responses in vegetative tissues. Roots of *CYP94B3-OE* seedlings were highly resistant to JA-induced growth inhibition (Fig. 3*D*). The level of JA insensitivity exhibited by *CYP94B3-OE* roots was slightly less than that of *coi1-1* seedlings (Fig. S5), indicating that CYP94B3 overexpression reduces but does not abolish responsiveness to exogenous JA. *CYP94B3-OE* seedlings were not affected in their responsiveness to the bacterial toxin coronatine (Fig. 3*E*), which is a potent agonist of the JA-Ile receptor (5, 24). These results indicate that reduced sensitivity of *CYP94B3-OE* plants to JA results from increased JA-Ile turnover rather than from a defect in hormone perception or signaling.

Leaves of *CYP94B3-OE* seedlings grown for extended periods (18 d) on JA-supplemented media also exhibited strong JA-resistant phenotypes, including loss of JA-induced chlorosis and growth stunting (Fig. 3*F*). To determine whether ectopic expression of CYP94B3 compromises anti-insect defense responses in leaves, we compared the performance of the generalist herbivore *Spodoptera exigua* on adult WT and transgenic plants. Insects reared on *CYP94B3-OE* plants for either 8 or 14 d were much heavier ($P < 0.0001$) than insects grown on the WT (Fig. 3*G*). The increased weight gain of larvae grown on the transgenic line was correlated with increased damage to *CYP94B3-OE* leaves, as well as reduced JA-Ile levels in insect-damaged leaf tissue (Fig. S6). Consistent with these results, JA-responsive transcripts accumulated to lower levels in mechanically wounded *CYP94B3-OE* leaves compared with WT leaves (Fig. S7*A*).

CYP94B3 Negatively Regulates Wound-Induced Gene Expression. The JA-insensitive phenotype of *CYP94B3-OE* plants indicated that CYP94B3 may act in WT plants to dampen jasmonate responses that are activated by stress-induced JA-Ile synthesis. To test this hypothesis, we compared WT and *cyp94b3* plants with respect to the wound-induced expression pattern of several primary response genes that are rapidly activated in response to increased JA-Ile levels (8, 9). We observed a pronounced effect of *cyp94b3-1* on *JAZ8* and *JAZ10* transcript levels, which hyperaccumulated in the mutant (relative to WT) during the 1- to 5-h time frame after wounding (Fig. 4*A*). Increased expression of *JAZ8* and *JAZ10* during this period correlated with increased JA-Ile content measured in the same tissue (Fig. 4*B*). Two independent experiments confirmed that *JAZ7*, *JAZ8*, and *JAZ10* mRNAs

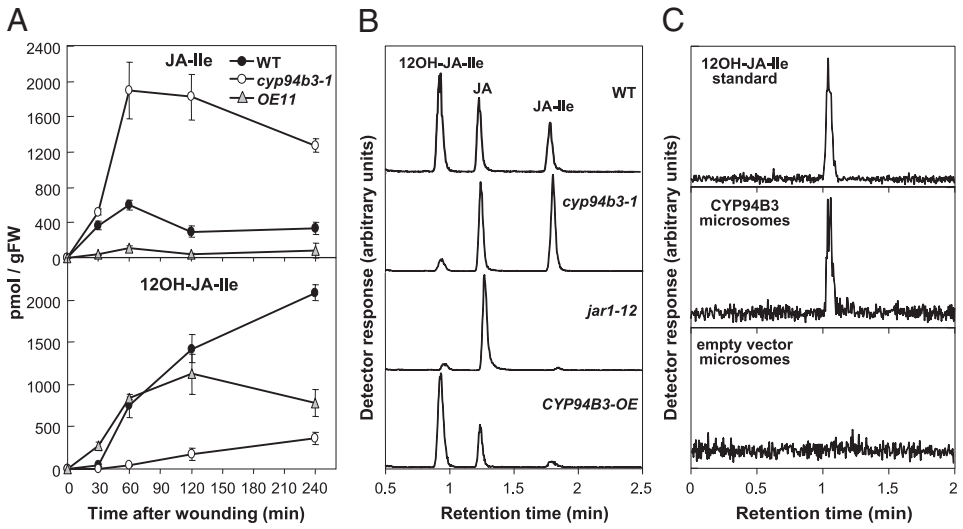

Fig. 2. *CYP94B3* encodes a JA-Ile-12-hydroxylase. (*A*) Time course of JA-Ile and 12OH-JA-Ile accumulation in wounded leaves of WT, T-DNA insertion knockout (*cyp94b3-1*), and CYP94B3-overexpressing (*CYP94B3-OE11*) plants. Mechanically damaged leaves were harvested for jasmonate extraction at various times after wounding for measurement of JA-Ile (*Upper*) and 12OH-JA-Ile (*Lower*). Each datapoint represents the mean ± SD of three biological replicates. (*B*) LC chromatogram of 12OH-JA-Ile, JA, and JA-Ile in wounded leaves (harvested 2 h after wounding) of WT, *cyp94b3-1*, *jar1-12*, and *CYP94B3-OE* plants. (*C*) Heterologously expressed CYP94B3 has JA-Ile-12-hydroxylase activity. Microsomal preparations from yeast transformed with either CYP94B3 or an empty vector control were incubated with JA-Ile for 1 h. Reaction products were analyzed by LC-MS/MS for the presence of a product whose retention time and mass spectrum (*m/z* 338 > 130) matched that of a 12OH-JA-Ile standard.

persist to higher levels in *cyp94b3* leaves than WT leaves during later stages of the wound response (Fig. S7).

12OH-JA-Ile Is Less Active than JA-Ile in Promoting COI1–JAZ Interaction. To investigate the signaling potential of 12OH-JA-Ile, we used in vitro pull-down assays to compare the ability of 12OH-JA-Ile and JA-Ile to promote interaction between COI1 and JAZ proteins. JA-Ile stimulated COI1 binding to full-length JAZ3 (JAZ3.1) in a dose-dependent manner with a stimulatory effect apparent at a concentration of 1 μM. 12OH-JA-Ile also promoted the COI1-JAZ3.1 interaction but, over the range of concentrations tested, was significantly and reproducibly less active than JA-Ile (Fig. 5*A*). Similar results were obtained in pull-down assays involving COI1 and full-length JAZ10 (JAZ10.1) (Fig. 5*B*).

Discussion

Elucidation of cellular processes governing JA-Ile homeostasis is essential for understanding developmental and defense-related processes mediated by this hormone. Here, we show that CYP94B3 is a JA-Ile-12-hydroxylase that defines a major pathway for JA-Ile catabolism. Overproduction of JA-Ile in wounded *cyp94b3* leaves indicates that this pathway operates as a negative feedback loop to effectively restrain JA-Ile accumulation. The time lag (~30 min) in wound-induced 12OH-JA-Ile accumulation relative to JA-Ile, which accumulates immediately upon leaf damage (8, 11, 25), is consistent with our in vivo and in vitro data showing that JA-Ile is a substrate for CYP94B3. The absence of 12OH-JA-Ile in undamaged WT and *CYP94B3-OE* leaves presumably reflects the lack of CYP94B3 substrate (JA-Ile) in these tissues. Given the wound-induced expression of *CYP94B3*, it is also possible that CYP94B3 activity is up-regulated during the wound response.

A key role for CYP94B3 in negative feedback control of JA-Ile production is supported by the fact that *CYP94B3* expression is positively regulated by COI1 and, consequently, that wounded *coi1* leaves hyperaccumulate JA-Ile. Low CYP94B3 abundance in *coi1* leaves is expected to slow the conversion of JA-Ile to 12OH-JA-Ile, thereby allowing JA-Ile to accumulate. This interpretation is consistent with studies showing that *coi1* mutants of tobacco and tomato also hyperaccumulate JA-Ile as a result of decreased JA-Ile turnover (23). The capacity of *coi1* plants to produce significant amounts of 12OH-JA-Ile (Fig. S1), however, indicates the existence of a COI1-independent route for 12OH-JA-Ile formation.

Cytochromes P450 comprise one of the largest and metabolically diverse protein families in the plant kingdom. Many P450s have well-established roles in phytohormone homeostasis, including CYP74s involved in JA biosynthesis and other aspects of oxylipin metabolism (26). Identification of CYP94B3 as a JA-Ile-12-hydroxylase extends the paradigm of P450-mediated oxidation as a general mechanism of hormone catabolism and, importantly, provides new insight into the function of a P450 family whose physiological role in plants remains largely unknown. Whereas *Arabidopsis* contains six CYP94s, the family has undergone significant expansion in other species; CYP94s comprise the largest non–A-type P450 family in soybean and rice, which have 14 and 18 members, respectively (27). *CYP94* genes are conserved in dicots, monocots, and nonvascular plants (e.g., *Physcomitrella patens*), but not in photosynthetic aquatic organisms (20, 28). This phylogenetic distribution highlights the importance of CYP94 in land plants and further suggests that pathways for jasmonate catabolism are conserved in evolution.

The role of CYP94B3 in JA-Ile hydroxylation indicates that other members of the CYP94 family may metabolize structurally related substrates. The fact that JA-Ile levels were not altered in *cyp94b1* and *cyp94c1* mutants indicates that these enzymes use other substrates or, alternatively, that they function redundantly with CYP94B3 in JA-Ile catabolism. The production of residual amounts of 12OH-JA-Ile in wounded *cyp94b3* leaves supports the existence of a JA-Ile-12-hydroxylase that is distinct from CYP94B3. CYP94s may also be involved in the synthesis of 12OH-JA, which accumulates to relatively high levels in wounded leaves (11, 13, 15, 16). Reduction in JA levels through conversion to 12OH-JA may provide an alternative pathway for limiting the production of bioactive JA-Ile (11, 13, 15), as could ω-hydroxylation of 12-oxo-phytodienoic acid or other JA precursors. The identification of 12OH-JAs as factors for leaf closing (17) and

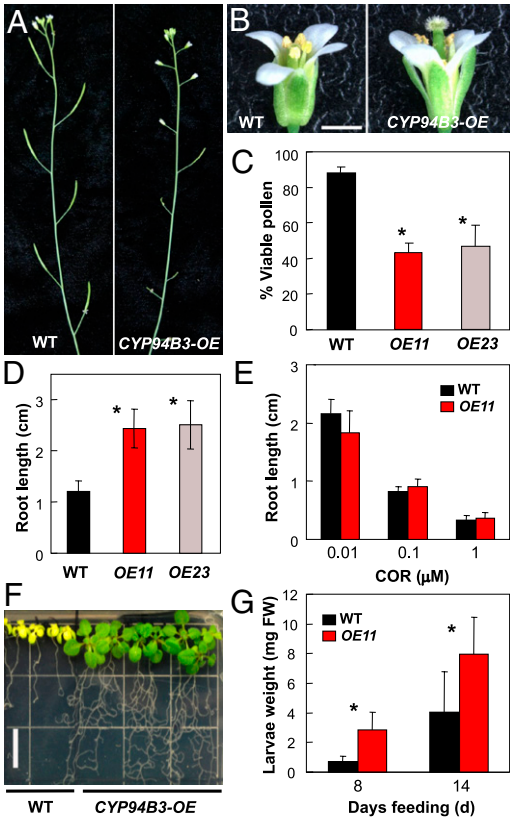

Fig. 3. Ectopic expression of CYP94B3 recapitulates JA-deficient phenotypes. (*A*) Photograph showing silique development in a WT (*Left*) and semisterile *CYP94B3-OE* (*Right*) plant. (*B*) Photograph of a representative flower from a WT (*Left*) and *CYP94B3-OE* (*Right*) plant. (Scale bar, 1 mm.) (*C*) Pollen viability in WT and two independent *CYP94B3-OE* lines (*OE11* and *OE23*). Pollen viability (200–600 pollen grains per plant) was assessed in five independent trials. Asterisks denote a statistically significant difference between WT and each transgenic line (*t* test, $P < 0.001$). (*D* and *E*) Root growth inhibition assays performed with WT and *CYP94B3-OE* (*OE11* and *OE23*) seedlings grown for 10 d on MS medium supplemented with either 10 μM JA (*D*) or varying concentrations of coronatine (COR) (*E*). Data show the mean ± SD ($n > 10$). In *D*, asterisks denote a significant difference between WT and each transgenic line (*t* test, $P < 0.001$). (*F*) Photograph showing that leaves of *CYP94B3-OE* seedlings grown for 18 d on media containing JA (10 μM) are insensitive to JA-induced growth inhibition and chlorosis. (Scale bar, 1 cm.) (*G*) *CYP94B3-OE* plants are compromised in resistance to attack by *S. exigua* larvae. Larvae were reared for 8 or 14 d on WT and *CYP94B3-OE* (*OE11*) plants. Values indicate the mean fresh weight ± SD of larvae ($n > 60$). Asterisks denote a significant difference between WT and transgenic host at each time point (*t* test, $P < 0.0001$).

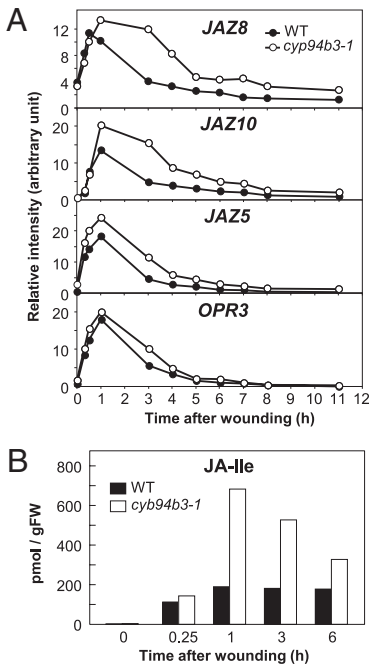

Fig. 4. Increased JA-Ile production in *cyp94b3* leaves is associated with enhanced expression of JA-responsive genes. (*A*) Time course of *JAZ* and *OPR3* mRNA accumulation in wounded leaves of WT and *cyp94b3-1* plants. RNA extracted from wounded and unwounded control (0 h time point) rosette leaves (30-d-old plants) was subjected to RNA blot analysis. Signal intensities were normalization to an *ACT8* loading control. (*B*) JA-Ile levels in leaf tissue from the same set of plants used in *A*. Each datapoint represents the mean of two biological replicates.

tuber induction (18) raises the possibility that CYP94s have a role in synthesizing biologically active jasmonates other than JA-Ile. Previous in vitro studies (19) showed that the JA-inducible CYP94C1 catalyzes ω- and in-chain hydroxylation of medium chain-length fatty acids. This enzyme also acts in vitro to convert 12OH-fatty acids to dicarboxylic derivatives. It is thus possible that CYP94s participate in the synthesis of 11OH-JA and dicarboxy-JA-Ile, both of which are produced in wounded leaves of *Arabidopsis* (11).

Overexpression of CYP94B3 resulted in several phenotypes typically observed in JA-deficient and JA-insensitive mutants of *Arabidopsis*. Based on the low level of JA-Ile in wounded *CYP94B3-OE* leaves and the in vitro activity of the enzyme, the most straightforward interpretation of this finding is that overexpression of CYP94B3 effectively depletes endogenous JA-Ile in roots, leaves, and flowers. Although we cannot exclude the possibility that CYP94B3 metabolizes other substrates (e.g., JA) in vivo, a key role for this P450 in JA-Ile turnover is consistent with the emerging view that JA-Ile is the active signal for many, if not most, jasmonate responses (1–5). For example, the increased susceptibility of *CYP94B3-OE* plants to attack by *S. exigua* larvae corroborates previous work showing that JA-Ile, rather than its metabolic precursors, is the relevant signal for induced resistance to lepidopteran insects (7, 29, 30). In the case of reproductive phenotypes, genetic studies have established a critical role for JA biosynthesis and perception in *Arabidopsis* male fertility (31). However, because *jar1* flowers are fully fertile, this mutant did not reveal a requirement for JA-Ile in fertility, most likely be-

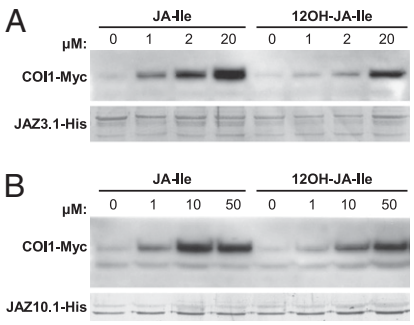

Fig. 5. 12OH-JA-Ile is less active than JA-Ile in promoting COI1 interaction with JAZ proteins. Pull-down assays were performed with the indicated concentration of JA-Ile or 12OH-JA-Ile, using Myc-tagged COI1 protein expressed in *Arabidopsis* and recombinant JAZ3.1-His (*A*) or JAZ10.1-His (*B*). Protein bound to JAZ-His was immunoblotted with anti-Myc antibody. As a loading control, the immunoblotted membrane was stained with Coomassie blue to show the recovery of the JAZ-His fusion protein.

cause JAR1-related enzymes produce JA-Ile to levels that are sufficient for fertility (25, 32). The male sterile phenotype of *CYP94B3-OE* plants helps to resolve this question by linking JA-Ile deficiency to specific defects in anther and pollen development. The strong JA-insensitive phenotype exhibited by *CYP94B3-OE* plants indicates that CYP94B3 overexpression provides a strategy to genetically ablate JA-Ile production without disrupting the jasmonate biosynthetic pathway.

There are several mechanisms by which CYP94B3 could inactivate JA-Ile signaling. One possibility is that 12OH-JA-Ile is not an effective ligand for the COI1-JAZ receptor. In support of this hypothesis, we found that 12OH-JA-Ile is significantly less active than JA-Ile in promoting COI1 binding to JAZ3.1 and JAZ10.1. Because the JA-Ile and 12OH-JA-Ile used for these experiments consisted of a mixture of stereoisomers (3), the biological significance of these in vitro results will require additional study. Recent work showing that JA-Ile acts more selectively in the nucleus than other jasmonate derivatives (33) raises the additional possibility that 12OH-JA-Ile does not partition effectively to the site of hormone perception in the nucleus. It is likely that CYP94B3-mediated inactivation of JA-Ile involves further enzymatic modification of 12OH-JA-Ile, for example by glucosylation, sulfation, or further oxidation of the 12-hydroxy group (11, 13, 16, 19). Such modifications are expected to restrict entry or otherwise modulate the manner in which the pentenyl side chain fits into the hydrophobic binding pocket of COI1 (5). Nevertheless, because 12OH-JA-Ile retains the ability to stimulate COI1-JAZ interaction in vitro, we cannot exclude the hypothesis that 12OH-JA-Ile associates with the receptor in vivo, possibly acting as a receptor agonist or antagonist. The *cyp94b3* mutants should be useful for future work aimed at studying the potential signaling role of 12OH-JAs.

The *cyp94b3* mutants also provide new tools to understand the physiological consequences of JA-Ile overproduction. A recent study reported that *cyp94b3* mutants have enhanced susceptibility to *Pseudomonas syringae* DC3000 (34). Based on the ability of the jasmonate pathway to suppress salicylate-based defenses against *P. syringae* (35), we suggest that increased JA-Ile levels in the infected mutant may contribute to pathogen virulence by suppressing anti-*P. syringae* defense responses. Unlike mutants that constitutively produce JA in the absence of stress (36), non-stressed *cyp94b3* plants do not exhibit obvious developmental or growth-related phenotypes. In mechanically damaged *cyp94b3* leaves, however, hyperaccumulation of JA-Ile resulted in the persistence of JA-responsive transcripts (e.g., *JAZ10*) at later stages of the wound response. This finding demonstrates that CYP94B3 has a physiological role in downregulating jasmonate responses in vegetative tissues, presumably as a mechanism to reduce fitness costs associated with overexpression of defensive traits (37). Effective attenuation of the jasmonate response is likely to involve not only JA-Ile catabolism, but also other mechanisms, including de novo synthesis of JAZ proteins that desensitize the JA-Ile receptor (38).

Materials and Methods

Plant Material and Growth Conditions. *Arabidopsis thaliana* ecotype Col-0 was used as the WT for all experiments. Plants were grown as previously described (9) or as described below for insect bioassays. T-DNA insertion lines (39) *jar1-12* (SALK_075487), *cyp94c1-1* (SALK_055455c), *cyp94b3-1* (CS302217), *cyp94b3-2* (SALK_018989c), and *cyp94b1-1* (SALK_129672) were obtained from the *Arabidopsis* Biological Resource Center (ABRC). Oligonucleotide primers used for plant genotyping are described in Fig. S2 and Table S2. For construction of the binary vector used to generate *CYP94B3-OE* lines, we used the primer pair JH1_XbaI F and JH1_XhoI R1 (Table S2) to PCR-amplify the full-length *CYP94B3* ORF from a cDNA clone (U64439) obtained from ABRC. The resulting PCR fragment was cloned into the XbaI and XhoI sites of a modified pBI121 vector (22), which places the gene under the control of the *cauliflower mosaic virus* 35S promoter. *Arabidopsis* was transformed with *Agrobacterium tumefaciens*, as previously described (9). Pollen viability was determined with the fluorescein diacetate/propidium iodide staining procedure (40).

Analytical Methods and Chemicals. Jasmonate measurements were performed by LC-MS/MS as previously described (9). Dihydro-JA (dh-JA) and [$^{13}C_6$]JA-Ile were used as internal standards for the quantification of JA, JA-Ile, and 12OH-JA-Ile. The transitions from deprotonated molecules to characteristic product ions were monitored in electrospray negative mode for JA (*m/z*, 209 → 59), dh-JA (211 → 59), JA-Ile (322 → 130), [$^{13}C_6$]JA-Ile (328 → 136), and 12OH-JA-Ile (338 → 130). Peak areas were integrated and the analytes were quantified on the basis of standard curves generated by comparing analyte responses to the corresponding internal standard. (±)-JA and coronatine were purchased from Sigma-Aldrich. The 12OH-JA-Ile was a gift from Paul Staswick (University of Nebraska, Lincoln, NE), and was chemically synthesized from 12OH-JA and Ile, as previously described (41). [$^{13}C_6$]JA-Ile and (–)-JA-Ile, which consists of a mixture of the (3R, 7R) and (3R, 7S) stereoisomers, were synthesized as described by Chung et al. (8).

Plant Treatments. Fully expanded rosette leaves of 4- to 5-wk-old plants were wounded across the midrib with a hemostat as previously described (9). At various times after wounding, damaged leaves were harvested, immediately frozen in liquid nitrogen, and stored at –80 °C until needed for RNA or jasmonate extraction. Root growth inhibition assays were performed on sucrose-containing (1%) MS medium as previously described (42). *S. exigua* eggs were obtained from Benzon Research and hatched at 30 °C. Newly hatched larvae were transferred to 5-wk-old plants grown in a growth chamber maintained at 21 °C under 12-h light (100 μE $m^{-2}·s^{-1}$) and 12-h dark. Eight to 10 larvae were caged on two plants of the same genotype grown in a single pot. The cage was constructed by placing an inverted clear plastic cup, in which the bottom was removed and covered with miracloth to allow air exchange, over the pot.

RNA Analysis. RNA extraction was performed with the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA blot analysis was conducted as described previously (22). Gene-specific probes were prepared by PCR amplification of the corresponding cDNA clones for *JAZ5*, *JAZ7*, *JAZ8*, *JAZ10*, *OPR3*, and *ACT8* (8, 9), or using genomic DNA as template for amplification of *CYP94C1*, *CYP94B3*, and *CYP94B1* (Table S2). Signal intensities on autoradiographs were quantified by densitometry (Quantity One software; Bio-Rad) and were normalized to values obtained for an *ACT8* loading control.

In Vitro Assays. A yeast heterologous expression system optimized for plant P450 enzymes (43) was used to express CYP94B3 for in vitro enzyme assays. The *CYP94B3* ORF was PCR amplified using a full-length cDNA clone (U64439) as template, and subsequently ligated into the EcoRI site of yeast expression vector pYeDP60 (see Table S2 for primers). The resulting vector was transformed into *S. cerevisiae* WAT11 strain. Culturing of yeast cells and preparation of microsomes was carried out as previously described (19, 43). Microsomal protein was quantified with BCA protein assay reagent (Pierce). In vitro hydroxylation assays were performed as described by Li-Beisson et al. (44), with minor modifications. JA-Ile substrate consisting of a mixture of the (3R, 7R) and (3R, 7S) stereoisomers in ethanol (8), was evaporated in a microfuge tube and redissolved in 2 μL DMSO. The standard reaction (0.1 mL) contained 20 mM sodium phosphate (pH 7.4), 2 mM NADPH, 6.7 mM glucose-6-phosphate, 0.4 units of glucose-6-phosphate dehydrogenase (Sigma), 500 μg yeast microsomal protein, and 20 μM JA-Ile substrate. The reaction was initiated by the addition of NADPH and was incubated at 30 °C for 1 h. The reaction was terminated by addition of 200 μL methanol containing an internal standard. The resulting methanolic mixture was directly analyzed for the presence of 12OH-JA-Ile by LC-MS/MS. Under the assay conditions used, the amount of JA-Ile converted to 12OH-JA-Ile was estimated to be less than 1% of the substrate added to the reaction. In vitro COI1-JAZ pull-down assays were performed with enantiomeric mixtures of chemically synthesized JA-Ile and 12OH-JA-Ile (8, 41), as previously described (38).

ACKNOWLEDGMENTS. We thank Darya Howell and Noor Kamila Ahmad Shafiai for technical assistance, Paul Staswick for providing jasmonic acid conjugates, and Joe Chappell for providing the yeast WAT11 strain; and the Arabidopsis Biological Resource Center for providing T-DNA insertion lines and cDNA clones, and Dan Jones and Xiaoli Gao in the Michigan State University Mass Spectrometry Facility. This work was supported by the National Institutes of Health Grant R01GM57795 and the Chemical Sciences, Geosciences and Biosciences Division, Office of Basic Energy Sciences, Office of Science, US Department of Energy Grant DE–FG02–91ER20021.

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