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Arabidopsis CYP94B3 Encodes Jasmonyl-L-Isoleucine 12-Hydroxylase, a Key Enzyme in the Oxidative Catabolism of Jasmonate

Naoki Kitaoka[1], Takuya Matsubara[1], Michio Sato[1], Kosaku Takahashi[1], Shinji Wakuta[2], Hiroshi Kawaide[3], Hirokazu Matsui[2], Kensuke Nabeta[1] and Hideyuki Matsuura[1,*]

[1]Laboratory of Bioorganic Chemistry, Division of Applied Bioscience, Research Faculty of Agriculture, Hokkaido University, Sapporo, 060-8589 Japan
[2]Laboratory of Biological Chemistry, Division of Applied Bioscience, Research Faculty of Agriculture, Hokkaido University, Sapporo, 060-8589 Japan
[3]Institute of Agriculture, Tokyo University of Agriculture and Technology (TUAT), Fuchu, Tokyo, 183-8509 Japan
*Corresponding author: E-mail, matsuura@chem.agr.hokudai.ac.jp; Fax, +81-11-706-2505.
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The hormonal action of jasmonate in plants is controlled by the precise balance between its biosynthesis and catabolism. It has been shown that jasmonyl-L-isoleucine (JA-Ile) is the bioactive form involved in the jasmonate-mediated signaling pathway. However, the catabolism of JA-Ile is poorly understood. Although a metabolite, 12-hydroxyJA-Ile, has been characterized, detailed functional studies of the compound and the enzyme that produces it have not been conducted. In this report, the kinetics of wound-induced accumulation of 12-hydroxyJA-Ile in plants were examined, and its involvement in the plant wound response is described. Candidate genes for the catabolic enzyme were narrowed down from 272 Arabidopsis Cyt P450 genes using Arabidopsis mutants. The candidate gene was functionally expressed in *Pichia pastoris* to reveal that *CYP94B3* encodes JA-Ile 12-hydroxylase. Expression analyses demonstrate that expression of *CYP94B3* is induced by wounding and shows specific activity toward JA-Ile. Plants grown in medium containing JA-Ile show higher sensitivity to JA-Ile in *cyp94b3* mutants than in wild-type plants. These results demonstrate that CYP94B3 plays a major regulatory role in controlling the level of JA-Ile in plants.

Keywords: *Arabidopsis thaliana* • Cyt P450 • Jasmonic acid • Jasmonyl isoleucine • *W*ounding response.

Abbreviations: BA, benzoic acid; JA, jasmonic acid; JA-Ile, jasmonyl-L-isoleucine; JAZ, jasmonate Zim-domain; MS/MS, tandem mass spectrometry; OPDA, 12-oxo-phytodienic acid; RT–PCR, reverse transcription–PCR; SA, salicylic acid; SRM, selected reaction monitoring; TOF, time of flight; UHPLC, ultra high-performance liquid chromatography.

Introduction

Throughout their life, plants use a variety of hormonal signals to adjust growth in response to developmental stages and external cues. It is generally accepted that jasmonic acid (JA) and its derivatives are important signaling compounds in plant development and stress response pathways, such as senescence (Ueda and Kato 1980), wound response (Farmer et al. 1992) and reproductive development (Koda and Okazawa 1988, Yoshihara et al. 1989, Falkenstein et al. 1991, McConn and Browse 1996). JA is synthesized from the α-linoleinic acid released by lipase activity; the intermediate is converted by sequential peroxidation, dehydration and cyclization to form 12-oxo-phytodienic acid (OPDA). OPDA is converted to JA by reduction and three β-oxidation steps that shorten the acid side chain. JA is enzymatically converted into numerous conjugates and derivatives including methyl jasmonate (Seo et al. 2001) and JA–amino acid conjugates (Staswick and Tiryaki 2004).

In recent studies, it has been demonstrated that JA–amino acid conjugation by JAR1 (Jasmonate-Resistant 1) is necessary for JA-mediated signal activation, and jasmonyl-L-isoleucine (JA-Ile) was recently found to be the active form of the hormone (Staswick and Tiryaki 2004). Jasmonate Zim-domain (JAZ) repressors are key regulators of jasmonate signaling (Chini et al. 2007, Thines et al. 2007). JA-Ile plays a crucial role in the acquired resistance of Arabidopsis plants as an activator of the interaction of two proteins, SCF^{COI1} E3 ubiquitin ligase and JAZ (Thines et al. 2007). Binding of SCF^{COI1} to JAZ is not activated by JA, methyl jasmonate or OPDA. However, after binding to JA-Ile, JAZ was ubiquitinated and degraded with the 26S proteasome to desuppress the jasmonate signaling pathway.

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The pathway generating 12-hydroxyJA, 12-*O*-β-D-glucopyranosylJA and 12-*O*-sulfonylJA is a major jasmonate catabolism pathway. Both 12-hydroxyJA and 12-*O*-D-β-glucopyranosylJA were isolated as tuber-inducing factors from the leaves of potato (*Solanum tuberosum* L.) (Koda and Okazawa 1988, Yoshihara et al. 1989). In our previous study, it was suggested that 12-*O*-D-β-glucopyranosylJA was generated from JA to transmit potato tuber induction signals. Miersch et al. (2008) reported that 12-hydroxyJA contributes to the down-regulation of the JA-mediated signaling pathway to suppress JA biosynthetic genes, such as *LOX*, *AOS* and *OPR*, that are JA inducible. The 12-hydroxyJA-Ile molecule is induced by wound response (Glauser et al. 2008), but its biological function is unknown. This compound was hypothesized to be synthesized from JA or JA-Ile via the oxidation of the 12-methyl carbon by an enzyme from the Cyt P450 family (**Fig. 1**). This family consists of a large and diverse group of heme-containing enzymes that are ubiquitous in bacteria, mammals, fungi and plants. Cyt P450s comprise about 1% of the genes in plants, such as Arabidopsis and rice. Hydroxylation is an important step in controlling plant hormone activities, such as gibberellin biosynthesis (CYP88A), brassinosteroid catabolism (CYP734A1) and JA biosynthesis (CYP74A) (Helliwell et al. 2001, Park et al. 2002, Turk et al. 2005). Catabolic inactivation of ABA is mainly controlled by ABA 8′-hydroxylase. This Cyt P450 catalyzes the C8′-hydroxylation of ABA to 8′-hydroxy-ABA and its more stable tautomer, phaseic acid, which has much lower hormonal activity than ABA. ABA 8′-hydroxylase has been identified as CYP707A1-4 in *Arabidopsis thaliana* (Kushiro et al. 2004, Saito et al. 2004); since its discovery, many CYP707A isozymes have been found in plants (Millar et al. 2006, Yang and Choi 2006, Yang and Zeevaart 2006). Gene knockdown and overexpression studies suggest that ABA 8′-hydroxylase is a key enzyme for controlling ABA concentration during water deficit stress or dormancy maintenance and breaking (Okamoto et al. 2006, Umezawa et al. 2006).

Hydroxylation of jasmonate is expected to be an important pathway in the adjustment of JA-mediated signal response. In particular, the catabolic pathway of JA-Ile is thought to be very important in the management of JA-Ile levels in plants and ultimately leads to stress reduction and/or development responses, such as senescence, wound response and reproductive development. In this report, we conducted identification and functional analyses of JA-Ile 12-hydroxylase to uncover its significance. Our extensive and systematic prediction led to the successful identification of *CYP94B3* as the JA-Ile 12-hydroxylase gene. Quite recently, Koo et al. (2011) also reported that *CYP94B3* encodes this enzyme. However, we demonstrate this biological function using other approaches and provide additional data demonstrating the wound-induced accumulation of 12-hydroxyJA-Ile in wounded leaves from *A. thaliana*, tobacco and tomato. Samples were analyzed by ultra high-performance liquid chromatography tandem mass spectrometry (UHPLC MS/MS) using stable isotope-labeled compounds as internal standards. We also provide data elucidating the substrate specificity of CYP94B3 protein and altered sensitivity in the root elongation of *cyp94b3 A. thaliana*.

Results

Kinetic analysis of wound-induced accumulation of 12-hydroxyJA-Ile in damaged leaves

The biological role of 12-hydroxyJA-Ile in the plant wound response was studied in *A. thaliana*, tobacco and tomato. Fully expanded young leaves (approximately 2–3 g) were used in wounding treatments, and endogenous 12-hydroxyJA-Ile levels were analyzed by UHPLC MS/MS run in the selected reaction monitoring (SRM) mode using 12-hydroxyJA-[$^{13}C_6$]Ile as an internal standard. The internal standard was synthesized according to Scheme 1. The scarce information concerning the 12-hydroxyJA-Ile involved in the stress response prompted us to evaluate endogenous 12-hydroxyJA-Ile levels. The leaves of *A. thaliana*, tobacco and tomato were nipped, and the damaged leaves were harvested 0.5, 1, 2 and 4 h after the wounding treatment. The endogenous amount of 12-hydroxyJA-Ile in undamaged plants at 0 min was used as a control. The kinetic wound-induced accumulations of 12-hydroxyJA-Ile in the damaged leaves are shown in **Fig. 2A–C**. In general, the endogenous amount of JA-Ile shows transient accumulation; maximum levels of JA-Ile were observed at 0.5 h (Sato et al. 2009). Significant changes ($P < 0.05$) in accumulation of 12-hydroxyJA-Ile were observed after 1 h in tomato, 2 h in *A. thaliana* and 4 h in tobacco. These far-ranging results suggest that 12-hydroxyJA-Ile does have a certain biological role in the response of plants to wounding. Usually, the endogenous amount of 12-hydroxyJA-Ile is measured as a relative amount (Glauser

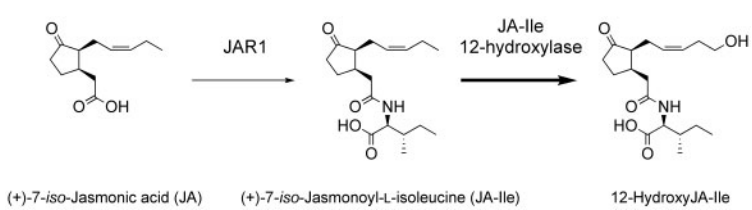
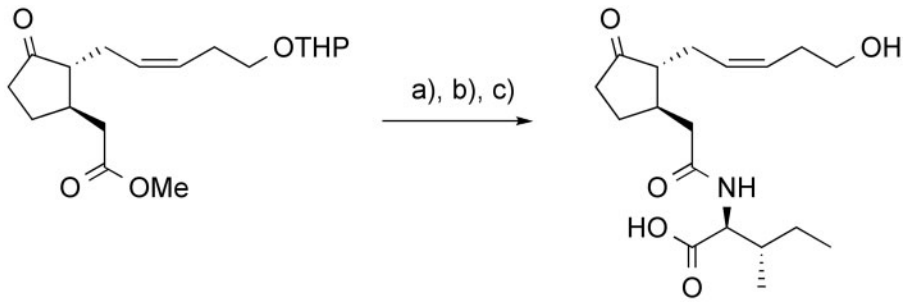


Fig. 1 Metabolism of JA-Ile. Jasmonic acid is activated by conjugation to isoleucine. The bold arrow shows the pathway from JA-Ile metabolism to 12-hydroxyJA-Ile by hydroxylation at the C-12 position.



Scheme 1 (a) NaOH/EtOH, (b) IR-120B/MeOH, (ci) isobutylchloroformate, Et_3N/THF, −40°C, (cii) L-Ile -UL-^{13}C /THF : H_2O (1 : 2), 1 M NaOH (22%, over three steps).

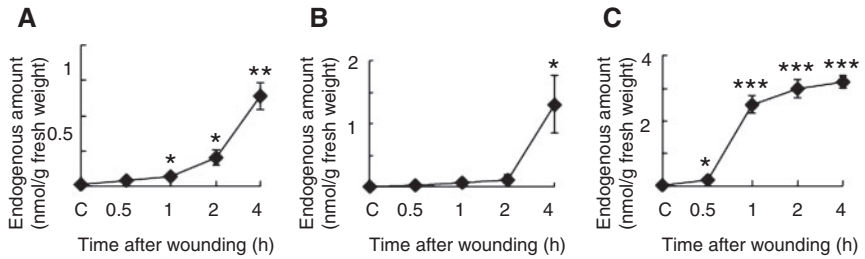


Fig. 2 Kinetics of wound-induced accumulation of 12-hydroxyJA-Ile in damaged leaves. Fully expanded younger leaves were mechanically damaged. The damaged leaves of *A. thaliana* (A), tobacco (*N. tabacum*) (*B*) and tomato (*S. lycopersicum*) (C) were harvested at the indicated time after the wounding treatment. The wound-induced accumulation of 12-hydroxyJA-Ile was analyzed by UHPLC MS/MS. C denotes control plant (no wounding, 0 h). Asterisks denote a significant difference between the sample and control using Student's *t*-test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

et al. 2008). Our measurement of the endogenous amount of 12-hydroxyJA-Ile using 12-hydroxyJA[$^{13}C_6$]Ile as internal standard is the first example of this.

P450 mutant screening and DNA microarray analysis of Arabidopsis P450s

There are 246 Cyt P450 genes and 26 related pseudogenes in the Arabidopsis genome. As previously predicted, jasmonate 12-hydroxylase is classified in the fatty acid ω-hydroxylase protein family due to its Cyt P450 enzymatic properties. Ehlting et al. (2008) and Narusaka et al. (2004) conducted microarray analyses of ω-hydroxylase Cyt P450 after wounding and other stresses and administering plant hormones. We narrowed down the candidates based on their data and monitored the wound-induced accumulation of JA-Ile and 12-hydroxyJA-Ile to identify the mutant *A. thaliana* plant carrying a disrupted JA-Ile 12-hydroxylase gene. The leaves were mechanically damaged and harvested 3 h after treatment. Endogenous levels of JA-Ile and 12-hydroxyJA-Ile in damaged leaves were measured (**Fig. 3A and B**, respectively). The *cyp94b3* mutant was determined to be the most promising candidate for disruption of JA-Ile 12-hydroxylase activity; its 12-hydroxyJA-Ile content was reduced to one-third of that of the wild type, while JA-Ile accumulated to levels five times higher than in the wild type. Wounding stress led to accumulation of the mRNA of the CYP94B3 gene (**Fig. 4**) as previously reported by Ehlting et al. (2008). Wound-induced accumulation of JA, JA-Ile, 12-hydroxyJA and 12-hydroxyJA-Ile is shown in **Fig. 5**.

Functional expression of CYP94B3 and its substrate specificity

Full-length cDNA for CYP94B3 was functionally expressed in *Pichia* transformants containing the Arabidopsis *CPR* (*ATR*) cDNA gene because NADPH-cytochrome P450 reductase (CPR) is indispensable in the maintenance of P450 enzyme activity. The cDNA of CYP94B3 was converted into pPICZ, and the vector was transformed into *Pichia* containing the *ATR* gene. *Pichia* transformants resistant to both blasticidin S and zeocin were obtained, and the transformants showing robust growth were further selected for use in heterologous expression. We tested JA-Ile 12-hydroxylase activity of the selected transformants. The cells of the *Pichia* transformant were incubated with JA-Ile, and 12-hydroxyJA-Ile levels in the supernatant were analyzed by UHPLC time of flight (TOF) MS using 12-hydroxyJA-[$^{13}C_6$]Ile as an internal standard (**Fig. 6**). While 12-hydroxyJA-Ile was detected when feeding JA-Ile to transformants containing ATR1 and CYP94B3, it was not detected in transformants containing only ATR1 (**Fig. 6**).

To study the substrate specificity of CYP94B3, the cells of the *Pichia* transformant were incubated with JA, JA-Leu and

Fig. 3 Levels of accumulation of JA-Ile and 12-hydroxyJA-Ile in CYP450 mutants in response to wounding. Fully expanded young *A. thaliana* leaves were mechanically damaged. The leaves were harvested 3 h after wounding. The wound-induced accumulation of JA-Ile and 12-hydroxyJA-Ile was analyzed by UHPLC MS/MS and is represented as a ratio of endogenous levels of JA-Ile (A) and 12-hydroxyJA-Ile (B) compared with the levels of wild-type seedling.

benzoic acid (BA). The products were evaluated using UPLC TOF MS. In the cases of JA and BA, the ions of the predicted products were not observed (**Table 1**). These results were further supported by UPLC MS/MS analysis run in the SRM mode. The ions of the predicted product of JA-Leu were observed in small amounts (0.3 nmol ml^{-1}), but incubation with JA-Ile gave product concentrations of 2.6 nmol ml^{-1}. This result indicates that the CYP94B3 protein expressed in the *Pichia* transformants has strict substrate specificity.

Phenotypic analysis of CYP94B3 knockout lines

To evaluate the in vivo function of CYP94B3 genes, we analyzed its corresponding insertion mutants. It is known that JA-Ile has inhibitory activity on root growth. The seeds of wild-type and *cyp94b3 A. thaliana* were sown in medium containing 50 μM JA-Ile and incubated for 10 d. We found that the root length of *cyp94b3 A. thaliana* was shorter than that of the wild type, and post-germination growth of the leaves of *cyp94b3 A. thaliana* displayed severe shrinkage (**Fig. 7**). This result suggests that disabling JA-Ile catabolism has an inhibitory effect on growth and that fine-tuning of JA-Ile levels is needed for normal growth.

Discussion

CYP94B3 is categorized into the CYP94 protein family, whose members mediate fatty acid ω-hydroxylation. In plants, fatty acid ω-hydroxylation is essential for the synthesis of the cuticle and is thought to be involved in plant signaling (Pinot and Beisson 2011); C16 and C18 fatty acids are potent substrates metabolized by fatty acid ω-hydroxylases such as CYP94A1 (Kandel et al. 2007). Members of the CYP94B family (CYP94B1, CYP94B2 and CYP94B3) have been found to be ω-hydroxylases after heterologous expression in yeast (Benveniste et al. 2006). However, it has been noted that the specific activity of these CYP94B representatives is very low toward C12:0, C14:0, C16:0 and C18:1 fatty acids. In the present study, CYP94B3 converted JA-Ile to 12-hydroxyJA-Ile (**Fig. 6**). When JA-Leu was used as substrate, the hydroxylation activity decreased. We also show that CYP94B3 is not able to convert JA into 12-hydroxyJA. The fact that CYP94B3 possesses strict substrate specificity is important if the enzyme regulates the level of JA-Ile, the true bioactive form for the JA-mediated signaling system. To the best of our knowledge, there are no reports of fatty acid ω-hydroxylases that convert this JA–amino acid conjugate. The microbial susceptibility of *cyb94b3 A. thaliana* has been reported (Hwang and Hwang 2010). In that report, *cyp94b3 A. thaliana* was more susceptible to *Pseudomonas syringae* pv. *tomato* DC3000 infection than the wild type. It is generally accepted that plants mainly show resistance to *Pst* DC3000 through a salicylic acid (SA) response, and it was assumed that SA-dependent signaling might be suppressed by accumulated JA-Ile in mutant plants, which acts as an antagonist.

Accumulated 12-hydroxyJA-Ile was observed in damaged leaves (**Fig. 2**). Wounding stress induced accumulation of CYP94B3 mRNA (**Fig. 4**), consistent with a previous report by Ehlting et al. (2008). These results reveal a role for CYP94B3 in plant wounding response. In post-germination growth in media containing JA-Ile, the root growth of *A. thaliana* was stagnant; *cyp94b3 A. thaliana* displayed more severe symptoms than the wild type. Severe inhibition of root growth is thought to result from overaccumulation of JA-Ile. Our results suggest that CYP94B3 maintains proper cellular levels of JA-Ile.

The work presented here demonstrates that *CYP94B3* codes for JA-Ile 12-hydroxylase. The enzyme seems to play a key role in JA-Ile catabolism and control of JA-Ile levels following various stress responses. Within the CYP94B family, two isozymes, CYP94B1 and CYP94B2, are most likely to play a similar role to CYP94B3. In this report, it was found that wound-induced accumulation of 12-hydroxyJA-Ile in *cyp94b1* and *cyp94b2* seedlings is slightly higher than in the wild type (**Fig. 3**). One of the

possible reasons was thought to be that these enzymes might convert 12-hydroxyJA-Ile to a further oxidized compound such as 12-carboxyjasmonoyl-L-isoleucine (Gaetan et al. 2008). This hypothesis was supported by the finding that CYP94C1 was involved in the formation of dicarboxylic fatty acids (Kandel et al. 2007). JA levels were higher in *cyp94b3* mutants than in the wild type (**Fig. 5**), and this increase may be due to up-regulation of JA biosynthesis by overaccumulated JA-Ile. Our present findings provide the framework for more extensive studies into the role and regulation of JA-Ile action during stress response and various plant life cycle stages. This information has the potential to have an enormous impact on the agricultural industry.

Materials and Methods

General

Electron impact (EI), field desorption (FD) and high-resolution field desorption (HRFD) mass spectra were obtained on a Jeol JMS-AX500 mass spectrometer. Column chromatography was performed with silica gel 60 (Spherical, 70–140 mesh, Kanto Chemical). UHPLC was performed on a Waters ACQUITY UHPLC system, equipped with a binary solvent delivery manager and a sample manager. MS was performed on a Waters Micromass' Quattro Premier tandem quadrupole mass spectrometer or a Waters LCT-Premier TOF mass spectrometer. The UHPLC/MS system control was by MassLynx 4.0. L-Isoleucine-UL-^{13}C was purchased from Medical Isotopes Inc.

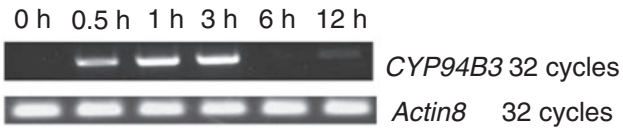


Fig. 4 Semi-quantitative RT–PCR analysis of *CYP94B3* transcription levels using total RNA extracted from 4-week-old *A. thaliana* wild-type seedlings after mechanical wounding with tweezers.

Synthesis of 12-hydroxyJA[$^{13}C_6$]Ile

A solution of 12-*O*-tetrapyranylmethyljasmonate (55 mg, 0.18 mmol), synthesized according to the reported method (Matsuura et al. 2000), was added to a stirred mixture of NaOH (100 mg) in anhydrous MeOH (3 ml). The reaction mixture was stirred for 24 h at room temperature. The reaction mixture was treated with IR-120B according to a standard method, and the volatile components of the mixture were evaporated in vacuo, resulting in a colorless oil (37 mg, 0.13 mmol). This product was conjugated with L-isoleucine-UL-^{13}C (58 mg, 0.42 mmol) according to the reported method (Sato et al. 2009) to give 12-hydroxyjasmonoyl-L-isoleucine-UL-^{13}C (12-hydroxyJA-[$^{13}C_6$]Ile, 14 mg, 0.04 mmol, 22%, over three steps): EI-MS *m/z* (relative intensity, %): 329 (15) [M-$^{13}CH_3$+H]$^+$, 299 (10), 275 (28), 193 (46), 167 (51) [M-CH_2COIle+H]$^+$, 152 (65), 91 (100). FD-MS *m/z* (relative intensity, %): 347 (15), 346 [M+H]$^+$ (100), 345 (37); HRFD-MS *m/z*: 346.2325 [M+H]$^+$ (calculated for $^{12}C_{12}{}^{13}C_6H_{30}O_5$: 346.2329).

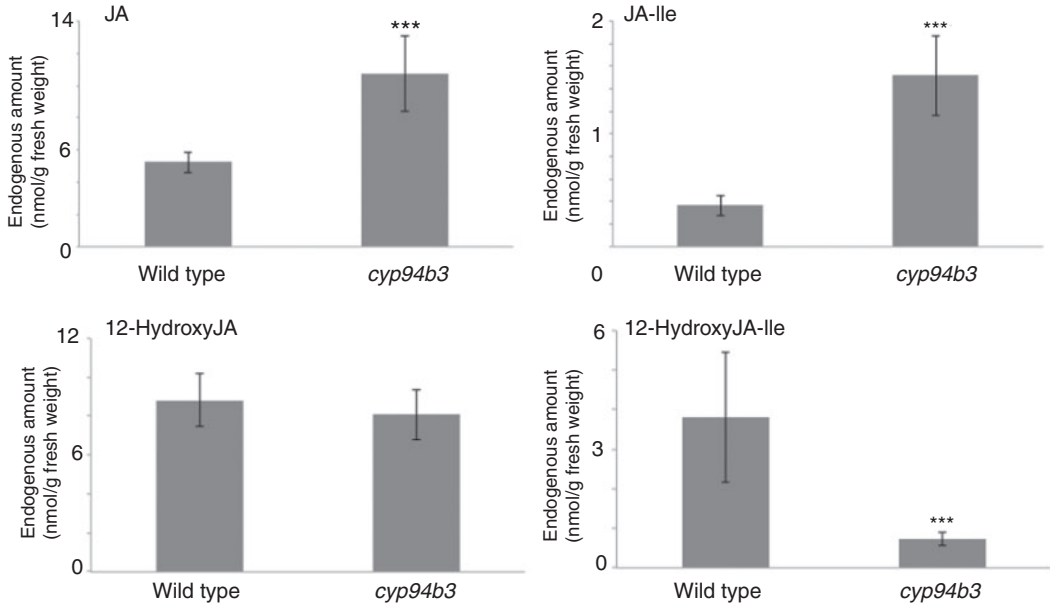


Fig. 5 The levels of JA, JA-Ile, 12-hydroxyJA and 12-hydroxyJA-Ile in the *cyp94b3* mutant following the wound response. Fully expanded young leaves of 4-week-old *A. thaliana* wild-type seedlings and *cyp94b3* mutants were mechanically damaged. The damaged leaves of *A. thaliana* were harvested 3 h after wounding. The wound-induced accumulation of JA, JA-Ile, 12-hydroxyJA and 12-hydroxyJA-Ile was analyzed by UHPLC MS/MS. Asterisks denote a significant difference between the mutant and wild type using Student's *t*-test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

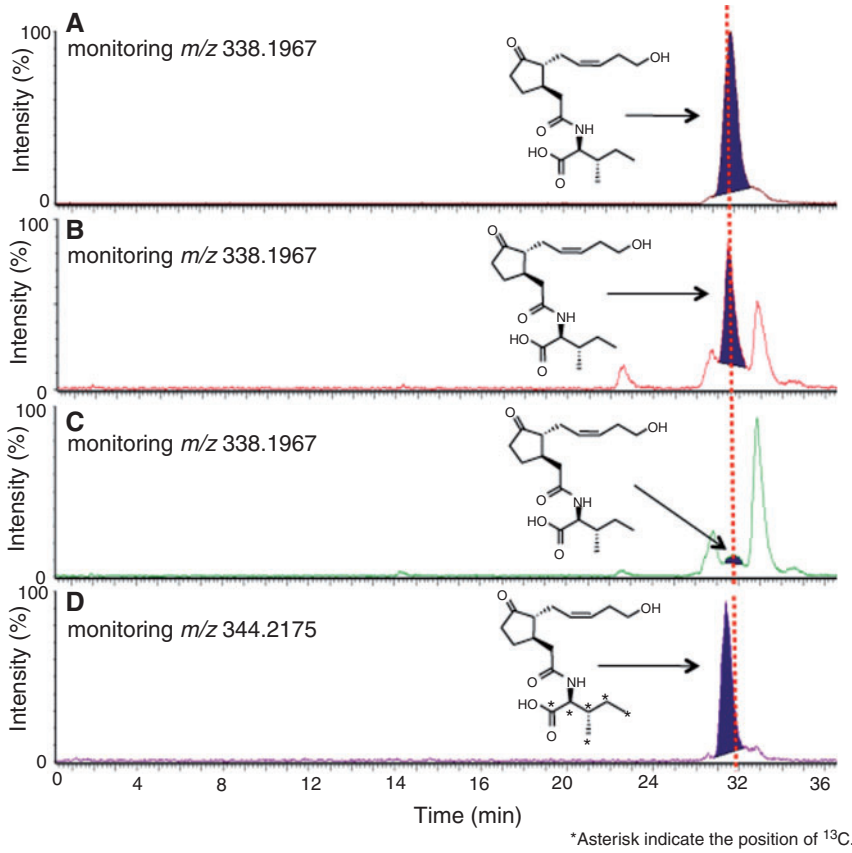


Fig. 6 In vivo formation of 12-hydroxyJA-Ile in *Pichia* co-expressing CYP94B3 and ATR1. Pichia transformed with either CYP94B3 or vector control were cultured in a medium contining JA-Ile (40 nmol). After 5 d, supernatant was analyzed by UHPLC TOF MS. The calculated precise molecular weights for $C_{18}H_{28}NO_5$ [12-hydroxyJA-Ile]$^{-1}$ and $^{12}C_{12}{}^{13}C_6H_{28}NO_5$ [12-hydroxyJA-[$^{13}C_6$]Ile]$^{-1}$ are *m/z* 338.1967 and 344.2175, respectively. (A) Synthetic 12-hydroxyJA-Ile; (B) and (C) 12-hydroxyJA-Ile-containing medium after feeding experiments using recombinant *Pichia* that do or do not express CYP94B3 with ATR1; (D) synthetic 12-hydroxyJA-[$^{13}C_6$]Ile.

Table 1 Substrate specificity of CYP94B3 fed with jasmonate and salicylic acid

Substrate	Relative activity (%)
JA-Ile	100
JA-Leu	12[a]
JA	s.l.[b]
BA	s.l.[c]

s.l., same level with and without CYP94B3.
[a] The predicted compound was 12-hydroxyJA-Leu.
[b] The predicted compound was 12-hydroxyJA.
[c] The predicted compound was hydroxyBA.

Plants and microorganisms

Plant materials used in this study were *A. thaliana* (L.) Heynh of ecotype Columbia, tobacco (*Nicotiana tabacum* cv. Xanthi nc) and tomato (*S. lycopersicum*). These seeds were planted into a Jiffy-7 (ϕ42 mm) pot, which had absorbed 50 ml of water, and the seedlings were grown in the growth chamber. The temperature and moisture of the chamber were set at 25°C and 60%, respectively, and the conditions of daylength were dark for 10 h and light for 14 h. Water was provided once a day, and a 500-fold diluted solution of Hyponex was added once a week as nutrition. Excess plants were removed in the interim such that there was only one plant per pot. The SALK T-DNA knock-out mutant lines were obtained from the Arabidopsis Biological Resource Center (ABRC: http://www.Arabidopsis.org/abrc/) at The Ohio State University (Columbus, OH). These stock names are shown in **Supplementary Table S1**. *Escherichia coli* strain JM109 was used for cloning and plasmid amplification.

Conditions for UPLC MS/MS

The conditions for UPLC MS/MS in SRM were set according to the reported method (Sato et al. 2011).

Conditions for UPLC TOF MS

The conditions for UPLC TOF MS were set according to the reported method (Sato et al., 2009) except for the following.

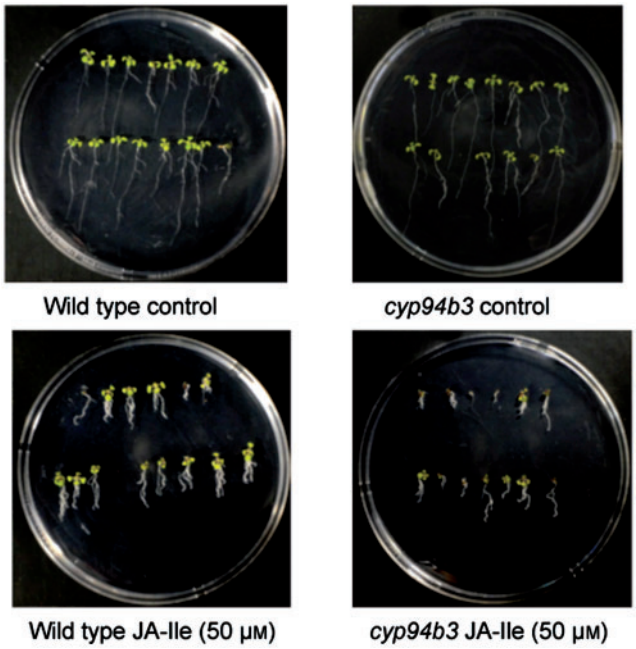


Fig. 7 Phenotypic analysis of the *cyp94b3* mutant. Root growth inhibition assay of 10-day-old *A. thaliana* wild-type seedlings and *cyp94b3* mutants grown with 50 μM JA-Ile.

The analytes were eluted from the column with a mixed solvent of 20% aqueous MeOH with 0.05% AcOH (solvent A) and MeOH with 0.05% AcOH (solvent B) using a liner gradient mode, in which the combination of A and B was 100 : 0 from 0 s to 0.2 min and, from 0.2 min to 57 min, the combination of A and B was linearly converted from 100 : 0 to 0 : 100. The combination of 0 : 100 was maintained from 57 min to 59 min. The total UPLC cycle time was 60 min including column re-equilibration. An eluent flow rate of 0.3 ml min^{-1} was employed for all analyses. Peak collection and statistical analysis of UPLC TOF MS peak data in each extracted mass chromatogram were integrated within a 0.05 Da mass window managed by MarkerLynx Application Manager version 4.1 (Waters).

Sample preparation of plant leaf materials for UPLC MS/MS and TOF MS analysis

The fully expanded younger leaves (approximately 2–3 g) of the plants were nipped carefully with tweezers to avoid the main veins. The damaged leaves were harvested after the indicated time. The leaves of unstressed plants were used as control. Plant material (2–3 g) was frozen using liquid nitrogen immediately after harvest. The frozen material was crushed and soaked in EtOH (20 ml) for 24 h. The mixture was filtered to result in a crude extract. Before purification, 12-hydroxyJA-[$^{13}C_6$]Ile (100 ng g^{-1}) was added to the extracts. The volatile components of the extract were removed under reduced pressure, and a solution of MeOH : H_2O (80 : 20, 2 ml) was added to the residue. The mixture was applied to a Bond Elut C_{18} cartridge column, and the column was successively washed with a solution of MeOH : H_2O (80 : 20, 2 ml × 2). The volatile components of the eluents were removed in vacuo, the residue was dissolved with a solution of MeOH : H_2O (80 : 20, 0.5 ml), and a portion of the mixture (5 μl) was subjected to UHPLC MS/MS. The conditions of UHPLC MS/MS were previously described (Sato et al. 2011), and the parameters for SRM analysis of 12-hydroxyJA-Ile and 12-hydroxyJA-[$^{13}C_6$]Ile were as follows: pseudo molecular ion $[M-H]^{-1}$, *m/z* 338.30 and 344.30; transition ion, *m/z* 129.66 and 135.66; cone voltage, 47 and 47 V; and collision energy, 23 and 23 eV, respectively.

cDNA cloning of CYP94B3

Arabidopsis leaves that were about 5 weeks old were wounded with tweezers. After 3 h, total RNA was extracted from the plants, and reverse transcription (Invitrogen, M-MLV reverse transcriptase) was conducted according to the manual to yield cDNA. PCR was performed with cDNA prepared from Arabidopsis leaf total RNA using a forward primer (CYP94B3-1F, 5′-GCATGGCATT TCTTCTGAGTTTTTTGAT A-3′) and a reverse primer (CYP94B3-1R, 5′-GCTTAAACGT T GTTAAGGATGTGACTTC-3′). The PCR was optimized for a 50 μl reaction mixture containing 0.2 μM of dNTP mix, 0.5 μM CYP94B3-1F, 0.5 μM CYP94B3-1R, 1 μl of cDNA, 25 μl of PCR buffer and 1 μl of KOD FX DNA polymerase (Toyobo). The reaction temperature was set to 98°C for 10 s, 55°C for 30 s and 68°C for 1 min 30 s, and repeated for 35 cycles. The PCR product was analyzed by gel electrophoresis and visualized using ethidium bromide staining. The gene fragment was cut from the gel and ligated into a pBluescript II SK cloning vector to obtain the plasmid pBS-CYP94B3.

Functional expression in *P. patoris*

After sequence analysis of pBS-CYP94B3, PCR was performed using primers containing a restriction enzyme site, forward primer (CYP94B3-2F with an *Eco*RI site, 5′-GCGAATTCATGG CATTTCTTCTGAGTTTTT-3′) and reverse primer (CYP94B3-2R with a *Kpn*I site, 5′-ATGGTACCTTAAACGTTGTTAAGGATG TGA-3′). The PCR protocol described above was used. After restriction with *Eco*RI and *Kpn*I, the gene fragment was ligated into the expression vector pPICZ-A, previously digested with *Eco*RI and *Kpn*I, to obtain the plasmid pPICZ-CYP94B3. The construct was transfected into *E. coli*, and the resulting plasmids were isolated and purified. The obtained plasmid was linearized with *Nsi*I, purified (5 μg $μl^{-1}$) and used to transform *Pichia*. We used *P. pastoris* containing the Arabidopsis P450 reductase gene, *ATR1* for transformation (Katsumata et al. 2008, Miyazaki et al. 2011). The linearized pPICZ-CYP94B3 gene was integrated into this transformant, and double transformants resistant to both blasticidin S and zeocin were selected

for on YPDS plates containing blasticidin S (300 μg ml^{-1}) and zeocin (100 μg ml^{-1}).

Heterologous production in *P. pastoris*

The transformation was incubated in 3 ml of minimal glycerol medium to increase cell numbers prior to MeOH induction. An induction culture for the production of CYP94B3 and ATR1 was continued in minimal MeOH medium according to the manufacturer's protocol. Induction was maintained by the addition of MeOH [final concentration 0.5% (v/v)] every 24 h. JA-Ile (40 nmol) was added to the cultures at the same time that MeOH induction was initiated. After 5 d of induction, the products were retrieved from the culture broth by centrifugation (3,000×*g*, 4°C, 10 min). An internal standard (12-hydroxyJA-[$^{13}C_6$]Ile; 0.5 nmol) was added to the supernatant before preparation. The supernatant was applied to the cartridge column of a Bond Elut C_{18}. The column was washed with water (2 ml × 3) and successively extracted with a solution of MeOH : H_2O (80 : 20, 2 ml × 3). The volatile eluent components were removed in vacuo, the residue was dissolved in a solution of MeOH : H_2O (80 : 200, 0.2 ml), and a portion of the mixture (5 μl) was subjected to UHPLC MS/MS using the conditions described above.

Semi-quantitative reverse transcription–PCR (RT–PCR) analysis of CYP94B3

Leaves of 5-week-old Arabidopsis plants were wounded with tweezers. After 0.5, 1, 3, 6 and 12 h, total RNA was extracted from the plant, and first-strand cDNA was synthesized with M-MLV reverse transcriptase (Invitrogen) according to the manual. Each PCR was performed in a 10 μl reaction mixture containing 0.2 μM of dNTP mix, 0.2 μl of first-strand cDNA, 5 μl of PCR buffer, 0.2 μl of KOD FX DNA polymerase (Toyobo) and 0.5 μM of gene-specific primers. The primers were as follows: CYP94B3, CYP94B3-1F, 5′-GCATGGCATTTCTTCTGAGTTTTTTGATA-3′ and CYP94B3-1R, 5′-GCTTAAACGTTGTTAAGGATGTGACTTC-3′; Actin8, act8-F, 5′-ATGGCCGATGCTGATGACAT TCAACCT-3′ and act8-R, 5′-TTAGAAGCATTTTCTGTGGACAATGCCTG-3′. The reverse-transcribed sample was first denatured at 94°C, and the PCR amplification was performed under the following conditions: 32 cycles of 98°C for 10 s, 55°C for 30 s and 6°C for 1 min 30 s. The PCR products were analyzed by gel electrophoresis and visualized using ethidium bromide staining.

Root length assay

Sterilized Arabidopsis seeds (Col-0) were plated on MS medium with and without 50 μM JA-Ile. Plates were incubated for 3 d at 4°C and grown for 9 d at 25°C.

Supplementary data

Supplementary data are available at PCP online.

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