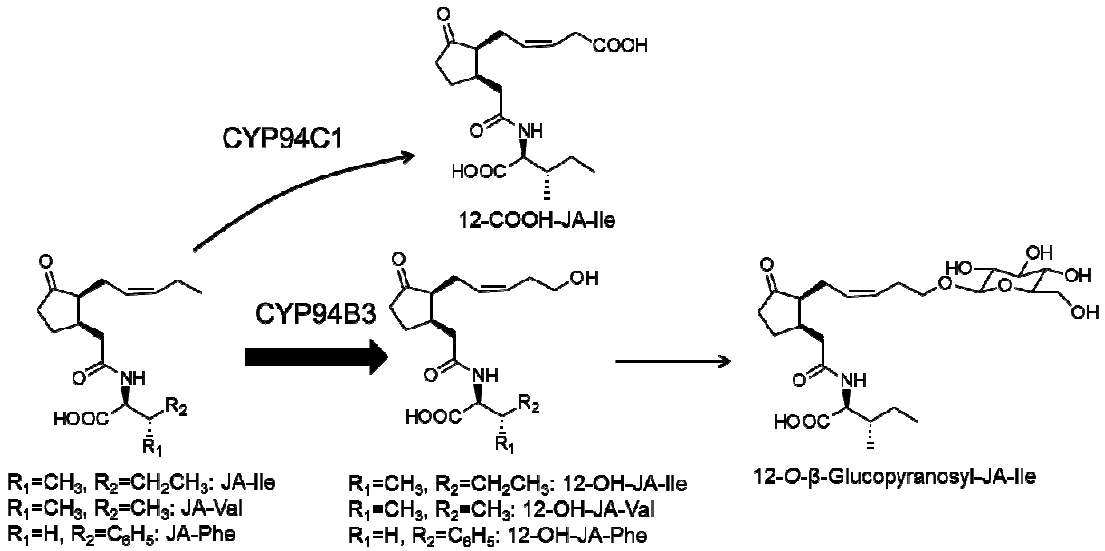
Graphical abstract



CYP94B3 activity against jasmonic acid amino acid conjugates and the elucidation of 12-*O*-β-glucopyranosyl-jasmonoyl-L-isoleucine as an additional metabolite

Naoki Kiatoka[a], Hiroshi Kawaide[b], Naruki Amano[a], Takuya Matsubara[a], Kensuke Nabeta[a], Kosaku Takahashi[a], and Hideyuki Matsuura[a,*]

[a] Laboratory of Bioorganic Chemistry, Division of Applied Bioscience, Research Faculty of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

[b] Institute of Agriculture, Tokyo University of Agriculture and Technology (TUAT), Fuchu, Tokyo 183-8509, Japan

*Corresponding author (Hideyuki Matsuura)

Tel: +81-11-706-2495

Fax: +81-11-706-2505

E-mail: matsuura@chem.agr.hokudai.ac.jp

Abstract

The hormonal action of jasmonate in plants is controlled by the precise balance between its biosynthesis and inactivation. The oxidation of jasmonoyl-L-isoleucine at the C-12 position, which is catalyzed by cytochrome P450s CYP94B3 and CYP94C1, is thought to be one of main inactivation pathways. In this study, we elucidated an additional function of CYP94B3 and investigated additional jasmonoyl-L-isoleucine metabolites. We found that CYP94B3 also catalyzes the hydroxylation of jasmonoyl-L-valine and jasmonoyl-L-phenylalanine, and these hydroxyl compounds accumulated after wounding and possessed lower activity than non-hydroxylated compounds. Additionally, 12-*O*-β-glucopyranosyl-jasmonoyl-L-isoleucine accumulated after wounding, suggesting that it is a metabolite of jasmonoyl-L-isoleucine.

Keywords:

Arabidopsis thaliana, *Glycine max*, *Nicotiana tabacum*, metabolic pathway, jasmonate, cytochrome P450, CYP94B3

Abbreviations

ESI, electrospray ionization; FD, field desorption; MS, mass spectrometry; MS/MS, tandem mass spectrometry; SRM, selected reaction monitoring; TOF, time of flight; UHPLC, ultra-high performance liquid chromatography

1. Introduction

Jasmonic acid (JA) and its derivatives regulate a wide range of biological processes, including plant defense, growth control, and reproductive development. The JA response is ingeniously controlled by many functions, such as biosynthesis, activation, signal transduction, transportation, and inactivation (Chung et al., 2009; Koo and Howe 2012; Schaller and Stintzi, 2009; Wasternack and Kombrinka, 2010). The biosynthesis of jasmonate is initiated by the oxygenation of α-linolenic acid in the chloroplast and terminates with synthesis of (+)-7-*iso*-JA in the peroxisome (Schaller and Stintzi, 2009). The synthesized (+)-7-*iso*-JA is metabolized to other jasmonates, including methyl jasmonate, 12-hydroxy-JA (12-OH-JA), and JA-amino acid conjugates (Wasternack and Kombrinka, 2010).

(+)-7-*iso*-Jasmonoyl-L-isoleucine (**1**, JA-Ile, Fig. 1), a dominant form of JA-amino acid conjugates, plays a crucial role as an activator of the interaction between two proteins, CORONATINE INSENSITIVE1 (COI1) and JASMONATEZIM-domain (JAZ), in *Arabidopsis* (Chini et al., 2007; Chung et al., 2009; Fonseca et al., 2009; Katsir et al., 2008; Thines et al., 2007; Yan et al., 2009). JAZ proteins are transcriptional repressors that, at low JA-Ile (**1**) concentrations, prevent the transcription of JA-responsive genes. In response to stress-related cues that trigger jasmonate accumulation, JA-Ile (**1**) stimulates the binding of JAZ to the E3 ubiquitin ligase SCF^{COI1}. The proteolytic degradation of JAZ via the ubiquitin/26S proteasome pathway releases JAZ-bound transcription factors from repression, thereby allowing the expression of JA-responsive genes. JA-amino acid conjugates such as JA-Ile (**1**) are synthesized by JAR1 (Jasmonate-Resistant 1) (Staswick and Tiryaki, 2004;

Suza and Staswick, 2008). Although JA-Ile (**1**) is the main amino acid conjugate in plant, the other conjugated forms of JA such as alanine (Ala), glutamine (Gln), leucine (Leu), valine (Val), phenylalanine (Phe), and threonine (Thr) have also been identified in wounded plants (Koo et al., 2009; Staswick and Tiryaki, 2004). The ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) conjugated to JA has been also found in plant tissue (Staswick and Tiryaki, 2004). While JAR1 has activity to produce several amino acids and ACC conjugates as described above, it has not completely clarified how these compounds were synthesized in plant. JA-Phe (**3**) and JA-ACC contents in *jar1* mutant suggest that at least one additional JA conjugating enzyme is present in Arabidopsis (Staswick and Tiryaki, 2004). JA-Val (**2**) also induces the interaction between COI1 and JAZ, like JA-Ile (**1**) does (Katsir et al., 2008). JA-tryptophan conjugate (JA-Trp) was reported to cause agravitropic root growth in seedlings with COI1 independent manner (Staswick, 2009). JA-Ile (**1**) has also been suggested to be a mobile signal from wounded leaves to other leaves (Matsuura et al., 2012; Sato et al., 2011).

The concentration of JA-Ile (**1**) is controlled by the precise balance between its biosynthesis and inactivation. There is detailed knowledge about nearly all genes that encode the core set of JA-Ile (**1**) biosynthetic enzymes, but the genetic basis of JA-Ile (**1**) inactivation is still a largely unexplored area. Recent studies have revealed that oxidation at the C-12 position of JA-Ile (**1**) by two cytochrome P450s, CYP94B3 and CYP94C1, plays an important role in jasmonate inactivation (Heitz et al., 2012; Kitaoka et al., 2011; Koo et al., 2011). CYP94B3 efficiently performs the initial hydroxylation of JA-Ile (**1**) to yield 12-OH-JA-Ile (**4**, Fig. 1), and CYP94C1 preferentially catalyzes the formation of the carboxy-derivative, 12-COOH-JA-Ile (**7**, Fig. 1). 12-OH-JA-Ile (**4**) is less active in promoting the COI1-JAZ interaction, and the *A. thaliana cyp94b3* mutant exhibits enhanced

expression of wound-responsive genes. It has also been reported that *cyp94b3* mutants are more susceptible to a virulent strain of *Pseudomonas syringae*, which may reflect the fact that increased signaling through the JA-Ile (**1**) receptor compromises host resistance to this pathogen by suppressing the salicylate-mediated branch of immunity (Hwang and Hwang, 2010). In addition to the oxidation at the C-12 position, the hydrolysis of Ile also contributes to the JA-Ile (**1**) inactivation pathway (Woldemariam et al., 2012).

12-OH-JA and its derivatives, such as 12-*O*-β-glucopyranosyl-JA, and 12-*O*-sulfonyl-JA, had been identified as jasmonate metabolites before the role of 12-OH-JA-Ile (**4**) was elucidated (Gidda et al., 2003; Koda and Okazawa 1988; Yoshihara et al., 1989). Both 12-OH-JA and 12-*O*-β-glucopyranosyl-JA were isolated as tuber-inducing factors from the leaves of potato plants (*Solanum tuberosum* L.) (Koda and Okazawa 1988; Yoshihara et al., 1989). Previous studies suggest that the presence of 12-OH-JA and its derivatives is common in higher plants (Miersch et al., 2008; Seto et al., 2009). Miersch et al. reported that 12-OH-JA contributes to the down-regulation of the JA-mediated signaling pathway to suppress JA biosynthetic genes, such as *LOX*, *AOS*, and *OPR*, that can be induced by JA (Miersch et al., 2008).

In this study, it was demonstrated that CYP94B3 could hydroxylate not only JA-Ile (**1**) but also JA-Val (**2**) and JA-Phe (**3**). This finding led us examine the biological properties of JA-Val (**2**), JA-Phe (**3**), 12-OH-JA-Val (**5**), and 12-OH-JA-Phe (**6**) to provide further information on the JA deactivation pathway. Additionally, we identified 12-COOH-JA-Ile (**7**) and 12-*O*-β-glucopyranosyl-JA-Ile (**8**) as candidates for additional metabolites of JA-Ile (**1**).

2. Results and discussion

2.1. CYP94B3 plays role in the hydroxylation of JA-Val (**2**) and JA-Phe (**3**)

The substrate specificity of CYP94B3 was evaluated using JA-Ile, JA-Leu, JA-Val, JA-Phe, JA-Gly, JA-Ala, JA-Trp, and JA-ACC conjugates, which have been reported in the wounded plant (Koo et al., 2009; Staswick and Tiryaki, 2004). However, we could not use JA-Thr and JA-Gln as substrate, because those compounds were not available by our chemical preparation. In medium containing each JA amino acid conjugate, *Pichia pastoris* transformats harboring *A. thaliana* cytochrome P450 reductase gene with/without *CYP94B3* were cultured, and 5 days later the expected products in the medium were detected by UHPLC TOF MS. When the transformants expressing *AtCYP94B3* were cultured in the presence of JA-Val (**2**) or JA-Phe (**3**), the expected hydroxylated products were detected (Table 1). The expected products could not be detected after these compounds were fed to transformats without *CYP94B3* and after JA-Gly, JA-Ala, JA-Trp, and JA-ACC were fed. These results suggest that CYP94B3 can hydroxylate not only JA-Ile (**1**) but also JA-Val (**2**) and JA-Phe (**3**).

2.2 Analysis of the content of 12-OH-JA amino acid conjugates in wounded plants

The enzyme activity of CYP94B3 suggested the existence of 12-OH-JA amino acid

conjugates in plant. The exact contents of 12-OH-JA-Val (**5**) and 12-OH-JA-Phe (**6**) in *A. thaliana* (wild type and *cyp94b3* mutant), *Nicotiana tabacum*, and *Glycine max* were analyzed by UHPLC MS/MS to find out the existence and confirm the role of CYP94B3 against JA-Val (**2**) and JA-Phe (**3**) (Fig. 2; Table 2). Fully expanded leaves were nipped with tweezers, and wounded leaves were collected at the indicated times after wounding. After extraction with ethanol, the endogenous 12-OH-JA-Val (**5**) and 12-OH-JA-Phe (**6**) levels were analyzed by UHPLC MS/MS in selected reaction monitoring (SRM) mode using 12-OH-JA-[$^{2}H_{8}$]Val (**9**) and 12-OH-JA-[$^{2}H_{5}$]Phe (**10**) as internal standards. 12-OH-JA-Val (**5**), 12-OH-JA-Phe (**6**), and the corresponding deuterium-labeled compounds were synthesized according to a previously reported method (Kitaoka et al., 2011). Significant peaks contributed to the introduced valine or phenylalanine were observed in ^{1}H-NMR and ^{13}C-NMR. In addition to 12-OH-JA-Ile (**4**), 12-OH-JA-Val (**5**) and 12-OH-JA-Phe (**6**) accumulated after wounding (Fig. 2). The 12-OH-JA-Ile (**4**), 12-OH-JA-Val (**5**), and 12-OH-JA-Phe (**6**) contents at 6 h after wounding in wild-type plants were 9800 pmol/g fresh weight, 690 pmol/g fresh weight, and 78 pmol/g fresh weight, respectively. The accumulated 12-OH-JA-Val (**5**) and 12-OH-JA-Phe (**6**) levels in wild-type plants were higher than those in mutant plants. Those results supported the hypothesis that CYP94B3 has activity against JA-Val (**2**) and JA-Phe (**3**) in plants (Table 1). As observed in *A. thaliana*, 12-OH-JA-Val (**5**) and 12-OH-JA-Phe (**6**) accumulated in *N. tabacum* and *G. max* (Table 2).

2.3. Biological activities of 12-OH-JA-Val (**5**) and 12-OH-JA-Phe (**6**)

Previous studies have demonstrated that JA-Val (**2**) and JA-Phe (**3**) can be induced by wounding (Koo et al., 2009) and that JA-Val (**2**) has almost same activity as JA-Ile (**1**) in the stimulation of the interaction between COI1 and JAZ (Katsir et al., 2008). We hypothesized that CYP94B3 played a key role in the inactivation of JA-Val (**2**) and JA-Phe (**3**), and evaluated these biological activities in *A. thaliana* using a root inhibition test, which has been reported to be occurred with COI1 dependent (Fonseca et al., 2009). The roots of plants that were grown in medium containing JA-Val (**2**) and JA-Phe (**3**) were shorter than the roots of control plants, even though the activities of these compounds were lower than that of JA-Ile (**1**) (Fig. 3). The 12-OH-JA-Val (**5**) activity was significantly lower than that of JA-Val (**2**), and the average root length of 12-OH-JA-Phe (**6**) was longer than that of JA-Phe (**3**) although there was no significant difference between JA-Phe (**3**) and 12-OH-JA-Phe (**6**). We hypothesized that hydroxylation at the C-12 position is a common mechanism to deactivate JA amino conjugates. But a doubt might remain because we used (+)-JA amino acid conjugates and (±)-12-OH-JA amino acid conjugates in this study. One previous report showed that (+)-JA-Ile had stronger activity than (-)-JA-Ile (Fonseca et al., 2009), and this suggests that (±)-12-OH-JA amino acid conjugates gave less effect than (+)-12-OH-JA amino acid conjugates. The other possible reason of lower activity of 12-OH-JA-Val (**5**) and 12-OH-JA-Phe (**6**) than JA-Val (**2**) and JA-Phe (**3**) might be derived from this points.

2.4. Chemical synthesis of the 12-COOH-JA-Ile (**7**) and 12-*O*-β-glucopyranosyl-JA-Ile (**8**) standards

The level of activity of 12-OH-JA-Ile (**4**) was lower than that of JA-Ile (**1**). However,

12-OH-JA-Ile (**4**) retained biological activity similar to that of JA-Ile (**1**) (Fig. 3; Koo et al., 2011), suggesting the importance of further metabolism. We chose 12-*O*-β-glucopyranosyl-JA-Ile (**7**) and 12-COOH-JA-Ile (**8**) as candidates for the main metabolites, and we determined the contents of these compounds after wounding. Although the existence of 12-COOH-JA-Ile (**7**) has been already reported (Glauser et al., 2008; Heitz et al., 2012), in this study, its exact content was determined using a synthetic internal standard. We prepared 12-COOH-JA-Ile (**7**) and 12-COOH-JA-[$^{2}H_{3}$]Leu (**11**) from 12-OH-JA-Ile (**4**) and 12-OH-JA-[$^{2}H_{3}$]Leu, respectively, by Jones oxidation (Scheme 1). Additional peak around 170 ppm was observed in ^{13}C-NMR spectrum of these synthesized compounds although there was no peak contributed to methylene next to hydroxyl group in ^{1}H-NMR, suggesting oxidizing to carboxy group at C12 position.

12-*O*-β-Glucopyranosyl-JA-Ile (**8**) was synthesized from 12-OH-JA-Ile (**4**) using the following procedures (Scheme 2). 12-OH-JA-Ile (**4**) was methylated with diazomethane. The resulting compound **12**, was coupled with α-D-tetraacetylglucopyranosyl bromide (**13**) using the Koenigs-Knorr reaction. Finally, the acetyl group and the methyl ester were successively deprotected using sodium methoxide and sodium hydroxide to give 12-*O*-β-glucopyranosyl-JA-Ile (**8**). The structure of 12-*O*-β-glucopyranosyl-JA-Ile (**8**) was confirmed by the appearance of peaks contributed to β-glucopyranosyl group. Because we could not synthesize a stable isotope-labeled form of 12-*O*-β-glucopyranosyl-JA-Ile (**8**), we used 12-*O*-β-glucopyranosyl-[$^{2}H_{5}$]JA as the internal standard in the UHPLC MS/MS analysis.

2.5. Accumulation of 12-COOH-JA-Ile (**7**) and 12-*O*-β-glucopyranosyl-JA-Ile (**8**) in

wounded *A. thaliana* (wild type and *cyp94b3* mutant), *N. tabacum*, and *G. max*.

The accumulation of 12-COOH-JA-Ile (**7**) and 12-*O*-β-glucopyranosyl-JA-Ile (**8**) in *A. thaliana* (wild type and *cyp94b3* mutant), *N. tabacum*, and *G. max* was analyzed using same methods as for 12-OH-JA-Val (**5**) and 12-OH-JA-Phe (**6**) (Fig. 4; Table 2). 12-COOH-JA-Ile (**7**) and 12-*O*-β-glucopyranosyl-JA-Ile (**8**) accumulated after wounding. The contents of 12-OH-JA-Ile (**4**), 12-COOH-JA-Ile (**7**), and 12-*O*-β-glucopyranosyl-JA-Ile (**8**) in wild-type *A. thaliana* were, respectively, 9813 pmol/g fresh weight, 1800 pmol/g fresh weight, and 11 pmol/g fresh weight at 6 h after wounding. Previous studies indicate that more JA-Ile (**1**) accumulates in *cyp94b3* mutant plants than in wild-type plants, whereas there is no significant difference between *cyp94c1* mutant and wild-type plants (Heitz et al., 2012; Kitaoka et al., 2011; Koo et al., 2011). The lower level of accumulation of 12-COOH-JA-Ile (**7**) relative to 12-OH-JA-Ile (**4**) suggests that 12-oxidation by CYP94C1 contributes less to the fine tuning of JA-Ile and has little impact on the JA-Ile level in *cyp94c1* mutants. The content of 12-*O*-β-glucopyranosyl-JA-Ile (**8**) in *cyp94b3* mutant plants was lower than that in wild-type plants, whereas the 12-COOH-JA-Ile (**7**) content in *cyp94b3* mutant plants was not significantly different from that in wild-type plants (Fig. 4). The decline in the 12-*O*-β-glucopyranosyl-JA-Ile (**8**) content in *cyp94b3* mutant plants indicates that 12-*O*-β-glucopyranosyl-JA-Ile (**8**) is synthesized from 12-OH-JA-Ile (**4**). 12-COOH-JA-Ile (**7**) profile in *cyp94b3* mutant was different from previous paper by Heitz et al. (Heitz et al., 2012). In their paper, the content of 12-COOH-JA-Ile (**7**) in *cyp94b3* was less than that in wild type. It might be come from difference of the growth condition, the plant age or mechanical wounding method. As observed in *A. thaliana*, 12-COOH-JA-Ile (**7**) and 12-*O*-β-glucopyranosyl-JA-Ile (**8**) accumulated in *N. tabacum* and *G. max* (Table 2).

However, the accumulation of 12-*O*-β-glucopyranosyl-JA-Ile (**8**) cannot fully explain the inactivation of JA-Ile (**1**) because the concentration of 12-*O*-β-glucopyranosyl-JA-Ile (**8**) was much lower than the concentration of 12-OH-JA-Ile (**4**). It is possible that 12-OH-JA-Ile (**4**) is metabolized to other compounds such as JA-Ile glucose conjugate that ester bound is formed between hydroxyl group on glucose and carboxyl group on JA-Ile (**1**) to deactivate completely JA-Ile (**1**) response. Another possibility is that 12-OH-JA-Ile (**4**) is transported into a cell organelle like a vacuole. The concentration of 12-*O*-β-glucopyranosyl-JA-Ile (**8**) in unwounded soybean plants is below the detection limit of UHPLC MS/MS, although previously studies have shown that the content of 12-*O*-β-glucopyranosyl-JA was very high in Leguminosae, even in unwounded plants (Miersch et al., 2008; Seto et al., 2009). High accumulation of 12-OH-JA and 12-*O*-β-glucopyranosyl-JA compare to JA-Ile (**1**), 12-OH-JA-Ile (**4**), 12-COOH-JA-Ile (**7**), and 12-*O*-β-glucopyranosyl-JA-Ile (**8**) suggests that JA was constantly synthesized and was metabolized not to JA-Ile but to 12-*O*-β-glucopyranosyl-JA through 12-OH-JA in Leguminosae. In unwounded *A. thaliana*, 12-*O*-sulfonyl-JA is accumulated in addition to 12-OH-JA and 12-*O*-β-glucopyranosyl-JA and the content of 12-*O*-sulfonyl-JA is higher than others (Miersch et al., 2008). This jasmonate concentration in intact *A. thaliana* plants implicates metabolite pathway to these compounds. The existence of 12-OH-JA-Val (**5**), 12-OH-JA-Phe (**6**), 12-COOH-JA-Ile (**7**), and 12-*O*-β-glucopyranosyl-JA-Ile (**8**) provided possibility that plants produced 12-COOH-JA-Val, 12-COOH-JA-Phe, 12-*O*-β-glucopyranosyl-JA-Val, and 12-*O*-β-glucopyranosyl-JA-Phe.

3. Conclusion

Previous studies have demonstrated that the oxidation of JA-Ile (**1**) to 12-OH-JA-Ile (**4**) and 12-COOH-JA-Ile (**7**) by CYP94B3 and CYP94C1 plays a crucial role in jasmonate inactivation (Heitz et al., 2012; Kitaoka et al., 2011; Koo et al., 2011). In this study, it was found that CYP94B3 also hydroxylates JA-Val (**2**) and JA-Phe (**3**). 12-OH-JA-Val (**5**) and 12-OH-JA-Phe (**6**) accumulated after wounding, and the *A. thaliana* root inhibition activities of these compounds were lower than those of the unhydroxylated compounds. In addition, 12-OH-JA-Ile (**4**) was further metabolized to other compounds, such as 12-*O*-β-glucopyranosyl-JA-Ile (**8**). However, the contents of these compounds were lower than that of 12-OH-JA-Ile (**4**). The result indicates that there exists a 12-OH-JA-Ile deactivation mechanism other than the one we hypothesized in this study.

4. Experimental

4.1 Instrumentation

FD MS was performed on a JEOL JMS-AX500 mass spectrometer. ESI MS and HRESI MS were performed on a JEOL JMS-T100LP mass spectrometer. Column chromatography was performed with silica gel 60 (Spherical, 70-140 mesh, Kanto Chemical). Ultra high-performance liquid chromatography (UHPLC) was performed using an ACQUITY^{TM} UHPLC^{TM} system (Waters) equipped with a binary solvent delivery manager and a sample manager. MS/MS was subsequently performed using a Micromass Quattro Premier tandem quadruple MS system (Waters). TOF MS was subsequently performed using an LCT-Premier time of flight mass spectrometer (Waters). The UHPLC/MS system was controlled using MassLynx 4.0 (Waters). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM-500 FT-NMR.

4.2 Plants and microorganisms

Plant seeds (*A. thaliana*, *G. max*, and *N. tabacum*) were planted in Jiffy-7 (φ 42 mm) pots that had absorbed 50 mL of water, and the seedlings were grown in a growth chamber. The temperature and moisture of the chamber were set to 25 °C and 60%, respectively, and the day-length conditions were dark for 10 h and light for 14 h. Water was provided once per day. Excess plants were removed in the interim such that there was only one plant per pot.

The SALK T-DNA knockout mutant line *cyp94b3* (SALK_001709C) was obtained from the Arabidopsis Biological Resource Center (ABRC: http://www.Arabidopsis.org/abrc/) at Ohio State University (Columbus, OH).

4.3 Synthesis of 12-OH-JA-Val (**5**) and 12-OH-JA-Phe (**6**)

A solution of methyl 12-*O*-tetrapyranyljasmonate (55 mg, 0.18 mmol) was added to a stirred mixture of NaOH (100 mg) in anhydrous MeOH (3 mL) and stirred for 24 h at room temperature. The reaction mixture was treated with IR-120B according to a standard method, and the volatile components in the mixture were evaporated *in vacuo*, resulting in a colorless oil. This product was conjugated with amino acids (L-Val, L-Phe, L-[$^{2}H_{8}$]Val, or [$^{2}H_{5}$]Phe) according to the reported method (Kitaoka et al., 2012) to give 12-OH-jasmonic acid amino acid conjugates.

12-OH-JA-Val (**5**)

FD MS *m/z* (rel. int., %): 375 (40), 327 (21), 326 $[M+H]^{+}$ (100), 325 $[M]^{+}$ (12), 43 (46).

UHPLC TOF MS: *m/z* 324.1790 $[M-H]^{-}$ (calcd. for $C_{17}H_{26}NO_{5}$: 324.1811).

^{1}H-NMR (270 MHz, $CDCl_{3}$) δ: 6.78 (*J* = 7.5 Hz, N*H*), 6.73 (*J* = 7.5 Hz, N*H*), 5.45 (2H, m, H-9, H-10), 4.38-4.31 (1H, m, H-2'), 3.67-3.60 (2H, m, C-12), 2.74-2.62 (1H, m), 2.46-1.83 (11H, m), 1.60-1.43 (1H, m), 0.94 (3H, d, *J* = 6.3 Hz, H-45'), 0.91 (3H, d, *J* = 6.6 Hz, H-5').

^{13}C-NMR (125 MHz, $CDCl_{3}$) δ: 219.5, 172.4, 172.4, 128.9, 128.5, 61.9, 54.0, 41.6, 38.9, 38.0, 30.8, 27.3, 25.6, 19.3, 18.0.

12-OH-JA-Phe (**6**)

FD MS *m/z* (rel. int., %): 375 (28), 374 $[M+H]^+$ (100), 357 (23).

UHPLC TOF MS: *m/z* 372.1816 $[M-H]^-$ (calcd. for $C_{17}H_{26}NO_5$: 372.1811).

^{1}H-NMR (270 MHz, $CDCl_3$) δ: 7.28-7.22 (2H, m, H-6'), 7.22 (1H, m, H-7'), 7.16 (2H, d, *J* = 12.6 Hz, H-5'), 6.74 (d, *J* = 8.0 Hz, N*H*), 6.60 (d, *J* = 8.0 Hz, N*H*), 5.50-5.30 (2H, m, H-9, H-10), 4.72 (1H, dd, *J* = 13.2, 7.9 Hz, H-2'), 3.61 (2H, t, *J* = 6.6 Hz, H-12), 3.25-3.19 (1H, m, H-3'a), 3.04-2.95 (1H, m, H-3'b), 2.59 (1H, m, H-2a), 2.36-1.76 (10H, m), 1.40-1.28 (1H, m).

^{13}C-NMR (125 MHz, $CDCl_3$) δ: 219.7, 172.6, 172.4, 173.6, 136.1, 129.2, 128.6, 128.4, 127.1, 61.9, 52.9, 41.1, 38.9, 38.2, 38.0, 37.3, 27.0, 25.4.

12-OH-JA-[$^2H_8$]Val (**9**)

FD MS *m/z* (rel. int., %): 375 (72), 374 (75), 357 (32), 334 $[M+H]^+$ (100), 333 $[M]^+$ (16).

UHPLC TOF MS: *m/z* 332.2307 $[M-H]^-$ (calcd. for $C_{17}{}^1H_{18}{}^2H_8NO_5$: 332.2313).

12-OH-JA-[$^2H_5$]Phe (**10**)

FD MS *m/z* (rel. int., %): 380 (25), 379 $[M+H]^+$ (100), 331 (16).

UHPLC TOF MS: *m/z* 377.2121 $[M-H]^-$ (calcd. for $C_{21}{}^1H_{21}{}^2H_5NO_5$: 377.2125).

4.4 Chemical synthesis of 12-COOH-JA-Ile (**7**)

Jones reagent (4 mL) was added to a stirred solution of 12-OH-JA-Ile (**4**, 87 mg, 0.26

mmol) in acetone (4 mL) at 4 °C. After stirring for 30 min at 4 °C, the reaction was quenched with propan-2-ol (5 mL). The reaction mixture was diluted with H_2O (50 mL) and extracted with ethyl acetate (50 mL x 3). The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (CC) (Si-60N 20 g, $CHCl_3$:MeOH:AcOH = 90:10:0.1) to yield 12-COOH-JA-Ile (**7**, 13 mg, 0.04 mmol, 14%). 12-COOH-JA-[$^2H_3$]Leu (**11**) was synthesized from 12-OH-JA-[$^2H_3$]Leu using same methods as above.

12-COOH-JA-Ile (**7**)

ESI MS *m/z* (rel. int., %) 353 (18), 352 (100, $[M-H]^-$), 308 (13).

HR ESI MS (ESI): *m/z* 352.1766 $[M-H]^-$ (calcd. for $C_{18}H_{26}NO_6$; 352.1760).

^{1}H-NMR (270 MHz, CD_3OD) δ: 5.64-5.55 (1H, m, H-10), 5.55-5.48 (1H, m, H-9), 4.36 (1H, dd, *J* = 5.7 Hz, H-2'), 3.10 (1H, d, *J* = 6.9 Hz, H-11), 2.65-2.57 (1H, m), 2.46-2.28 (2H, m, H-8), 2.43-2.24 (4H, m), 2.18-2.00 (2H, m) 2.01-1.91 (1H, m), 1.92-1.84 (1H, m, H-3'), 1.60-1.46 (1H, m, H-5'a), 1.29-1.19 (1H, m, H-5'b), 0.95 (3H, d, *J* = 6.6 Hz, H-4'), 0.92 (3H, t, *J* = 7.3 Hz, H-6').

^{13}C-NMR (125 MHz, $CDCl_3$) δ: 220.2, 174.3, 173.6, 173.0, 129.0, 123.3, 56.9, 53.9, 39.9, 38.4, 37.1, 36.8, 32.3, 26.6, 25.0, 24.9, 14.7, 10.7.

12-COOH-JA-[$^2H_3$]Leu (**11**)

ESI MS *m/z* (rel. int., %) 358 (25), 357 (100, $[M+H]^+$), 311 (12).

HR ESI MS (ESI): *m/z* 355.1938 $[M-H]^-$ (calcd. for $C_{18}{}^1H_{23}{}^2H_3NO_6$; 355.1948).

^{1}H-NMR (270 MHz, Acetone-d_6) δ: 7.42 (1H, m), 5.56 (2H, m), 4.53 (1H, m), 3.15 (2H, d, *J* = 6.6 Hz), 2.60 (1H, m), 2.42-1.91 (7H, m), 1.81-1.50 (3H, m), 0.93 (3H, m).

4.5 Chemical synthesis of 12-*O*-β-glucopyranosyl-JA-Ile (**8**)

Diazomethane (2 mL) was added to a MeOH solution (3 mL) of 12-OH-JA-Ile (**4**, 95 mg, 0.27 mmol) at 4 °C. The reaction was quenched with acetic acid after 30 min. The reaction mixture was concentrated *in vacuo* to yield compound **12**.

Under N_2, compound **12**, HgO (146 mg, 0.68 mmol), $HgBr_2$ (145 mg, 0.41 mmol), and Drierite (145 mg) were dissolved in CH_2Cl_2 (10 mL), and then α-tetraacetylglucopyranosyl bromide (**13**, 446 mg, 1.09 mmol) in CH_2Cl_2 (4 mL) was added at room temperature. After stirring for 2 h, the temperature was increased to 35 °C. After stirring overnight, the reaction mixture was filtered with Celite and evaporated. The residue was purified by silica gel CC (Si-60N 20 g, EtOAc: *n*-hexane=4:1) to yield **14** (10 mg, 0.015 mmol, 6%).

Compound **14**

FD MS *m/z* (rel. int., %) 685 (45), 684 (98), 683 (100, $[M]^+$).

^{1}H-NMR (270 MHz, $CDCl_3$) δ: 6.11 (1H, d, *J* = 8.5 Hz), 5.40 (2H, m), 5.17 (1H, dd, *J* = 9.6, 9.6 Hz), 5.07 (1H, d, *J* = 9.9 Hz), 4.95 (1H, dd, *J* = 9.6, 8.1 Hz), 4.57 (1H, dd, *J* = 8.2, 4.9 Hz), 4.49 (1H, d, *J* = 7.9 Hz), 4.24 (1H, dd, *J* = 12.3, 4.8 Hz), 4.09 (1H, dd, *J* = 12.3, 2.1 Hz), 3.86 (1H, m), 3.96-3.61 (2H, m), 3.71 (3H, s), 3.50-3.42 (1H, m), 2.60 (1H, m), 2.42-1.81 (9H, m), 2.06 (3H, s), 2.02 (3H, s), 1.99 (3H, s), 1.97 (3H, s), 1.62-1.40 (2H, m), 1.31-1.12 (2H, m), 0.97-0.88 (6H, m).

Under N_2, compound **14** (10 mg, 0.015 mmol) and sodium methoxide (3 mg, 0.060 mmol) were dissolved in MeOH (2 mL) at 4 °C. After stirring for 2 h at 4 °C, the reaction

mixture was quenched with a small amount of dry ice and concentrated *in vacuo*. The residue was purified by silica gel CC (Si-60N 10 g, MeOH:$CHCl_3$=9:1) to yield **15** (7 mg, 0.013 mmol, 87%).

To a solution of **15** (7 mg, 0.013 mmol) in 80% EtOH/H_2O (600 µL) was added 1 M NaOH (13 µL, 0.013 mmol). After stirring at room temperature for 2 h, the reaction mixture was neutralized with Amberlite IR-120 (H^+) and concentrated *in vacuo*. The residue was purified by silica gel CC (Si-60N 10 g, MeOH:$CHCl_3$:AcOH = 70:30:0.1) to yield 12-*O*-β-glucopyranosyl-JA-Ile (**8**, 6 mg, 0.012 mmol, 92%).

12-*O*-β-glucopyranosyl-JA-Ile (**8**)

ESI MS *m/z* (rel. int., %) 501 (23), 500 (100, $[M-H]^-$).

HR ESI MS (ESI): *m/z* 500.2504 $[M-H]^-$ (calcd. for $C_{24}H_{38}NO_{10}$; 500.2501).

^{1}H-NMR (500 MHz, CD_3OD) δ: 5.52-5.46 (1H, m, H-10), 5.46-5.38 (1H, m, H-9), 4.26 (1H, d, *J* = 7.9 Hz, H-1”), 4.22 (1H, m, H-2’), 3.90-3.81 (2H, m, H-5’a, H-6’a), 3.68-3.61 (1H, m), 3.58-3.52 (1H, m), 3.36-3.31 (1H, m), 3.27-3.24 (2H, m, H-4”, H-5”b), 3.15 (1H, dd, *J* = 13.2, 4.1 Hz, H-1”), 2.43-2.24 (7H, m), 2.18-1.93 (3H, m), 1.91-1.81 (1H, m, H-3’), 1.59-1.49 (1H, m, H-5’a), 1.22-1.13 (1H, m, H-5’b), 0.93 (3H, d, *J* = 6.9 Hz, H-4’), 0.90 (3H, t, *J* = 7.6 Hz, H-6’).

^{13}C-NMR (125 MHz, CD_3OD) δ: 226.9, 174.5, 174.5, 128.0, 127.7, 102.1, 75.8, 75.7, 73.0, 69.7, 69.5, 60.6, 54.0, 48.7, 40.3, 38.0, 37.9, 36.6, 27.3, 26.5, 25.0, 24.6, 15.2, 10.7.

4.6 Heterologous production in *Pichia pastoris*

Pichia pastoris transformants expressing CYP94B3 and the *Arabidopsis* NADPH-cytochrome P450 reductase (CPR) were constructed using previously described methods. The transformants were incubated in 3 mL of minimal glycerol medium to increase the number of cells prior to MeOH induction. An induction culture for the production of CYP94B3 and ATR1 was grown 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. The JA amino acid conjugate (40 nmol) was added to the cultures at the same time that MeOH induction was initiated. After five days of induction, the products were retrieved from the culture broth by centrifugation (3000 x *g*, 4 °C, 10 min). The supernatant was applied to a Bond Elut C_{18} cartridge column. The column was washed with water (2 mL x 3) and successively extracted with a solution of MeOH:H_2O (80:20, 2 mL x 3). The volatile eluent components were removed *in vacuo*, the residue was dissolved in a solution of MeOH:H_2O (80:20, 0.2 mL), and a portion of the mixture (5 μL) was subjected to UHPLC TOF MS.

4.7 Preparation of leaf samples for UHPLC MS/MS and TOF MS analysis

Fully expanded young leaves (ca. 100 mg) of the plants were nipped with tweezers. The damaged leaves were harvested after the indicated times. The leaves of unstressed plants were used as a control. Plant material (ca. 100 mg) was frozen using liquid nitrogen immediately after harvest. The frozen material was crushed and soaked in EtOH (20 mL) for 16 h. The mixture was filtered to give a crude extract. Before purification, the following masses of JA derivatives were added to the extracts: 12-OH-JA-[$^{13}C_6$]Ile (10 ng),

12-OH-JA-[$^{2}H_{8}$]Val (10 ng), 12-OH-JA-[$^{2}H_{5}$]Phe (10 ng), 12-COOH-JA-[$^{2}H_{3}$]Leu (10 ng), and 12-*O*-β-glucopyranosyl-[$^{2}H_{5}$]JA (200 ng). The volatile components in 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 eluted with a solution of MeOH:H_2O (80:20, 2 mL x 2). The volatile components of the eluates were removed *in vacuo*, the residue was dissolved in 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 UHPLC condition were as previously described (Sato et al., 2009). The parameters for the SRM analysis of 12-OH-JA-Ile (**4**), 12-OH-JA-[$^{13}C_{6}$]Ile, and 12-*O*-β-glucopyranosyl-[$^{2}H_{5}$]JA were as previously described (Kitaoka et al., 2011; Seto et al., 2009), and the parameters for the SRM analysis of 12-OH-JA-Val (**5**), 12-OH-JA-Phe (**6**), 12-COOH-JA-Ile (**7**), 12-*O*-β-glucopyranosyl-JA-Ile (**8**), 12-OH-JA-[$^{2}H_{8}$]Val (**9**), 12-OH-JA-[$^{2}H_{5}$]Phe (**10**), and 12-COOH-JA-[$^{2}H_{3}$]Leu (**11**) were as follows: 12-OH-JA-Val (**5**) {[M-H]$^{-1}$: 324.18, transition ion (*m/z*): 115.58, cone voltage: 47 V, collision energy: 23 eV};12-OH-JA-Phe (**6**) {[M-H]$^{-1}$: 372.21, transition ion (*m/z*): 163.77, cone voltage: 47 V, collision energy: 23 eV}; 12-COOH-JA-Ile (**7**) {[M-H]$^{-1}$: 352.24, transition ion (*m/z*): 129.70, cone voltage: 39 V, collision energy: 28 eV}; 12-*O*-β-glucopyranosyl-JA-Ile (**8**) {[M-H]$^{-1}$: 500.31, transition ion (*m/z*): 129.70, cone voltage: 42 V, collision energy: 34 eV}; 12-OH-JA-[$^{2}H_{8}$]Val (**9**) {[M-H]$^{-1}$: 332.18, transition ion (*m/z*): 123.58, cone voltage: 47 V, collision energy: 23 eV}; 12-OH-JA-[$^{2}H_{5}$]Phe (**10**) {[M-H]$^{-1}$: 377.21, transition ion (*m/z*): 168.77, cone voltage: 47 V, collision energy: 23 eV}; and 12-COOH-JA-[$^{2}H_{3}$]Leu (**11**) {[M-H]$^{-1}$: 355.24, transition ion (*m/z*): 132.70, cone voltage: 39 V, collision energy: 28 eV}.

4.8. Root length assay

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

Acknowledgments

The authors are grateful to Mr. Kenji Watanabe, Mr. Yusuke Takada, and Dr. Eri Fukushi (Faculty of Agriculture, Hokkaido University) for obtaining the spectroscopic data for the experimental compounds. N.K. is the recipient of a JSPS Fellowship.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://xxxxxxxxxxxxxxxxxx.

References

Chini, A., Fonseca, S., Fernández, G., Adie, B., Chico, J.M., Lorenzo, O., Garcia-Casado, G., Lopez-Vidriero, I., Lozano, F.M., Ponce, M.R., Micol, J. L., Solano, R., 2007. The JAZ family of repressors is the missing link in jasmonate signaling. Nature 448, 666-671.

Chung, H.S., Niu, Y., Browse, J., Howe, G.A., 2009. Top hits in contemporary JAZ: An update on jasmonate signaling. Phytochemistry 70, 1547-1559.

Fonseca, S., Chini, A., Hamberg, M., Adie, B., Porzel, A., Kramell, R., Miersh, O., Wasternack, C., Solano, R., 2009. (+)-7-*iso*-Jasmonoyl-L-isoleucine is the endogenous bioactive jasmonate. Nat. Chem. Biol. 5, 344-350.

Glauser, G., Grata, E., Dubugnon, L., Rudaz, S., Farmer, E.E., Wolfender, J.L., 2008. Spatial and temporal dynamics of jasmonate synthesis and accumulation in *Arabidopsis* in response to wounding. J. Biol. Chem. 283, 16400-16407.

Gidda, S. K., Miersch, O., Levitin, A., Schmidt, J., Wasternack, C., Varin, L., 2003. Biochemical and molecular characterization of a hydroxyjasmonate sulfotransferase from *Arabidopsis thaliana*. J. Biol. Chem. 278, 17895–17900.

Heitz, T., Widemann, E., Lugan, R., Miersch, L., Ullmann, P., Désaubry, L., Holder, E., Grausem, B., Kandel, S., Miersch, M., Werck-Reichhart, D., Pinot, F., 2012. Cytochrome P450 CYP94C1 and CYP94B3 catalyze two successive oxidation steps of plant hormone jasmonoyl-isoleucine for catabolic turnover. J. Biol. Chem. 287, 6296-6306.

Hwang, I.S. and Hwang, B.K., 2010. Role of the pepper cytochrome P450 gene *CaCYP450A* in defense responses against microbial pathogens. Planta 232, 1409-1421.

Katsir, L., Schilmiler, A.L., Staswick, P.E., He, S.Y., Howe, G.A., 2008. COI1 is a critical component of a receptor for jasmonate and bacterial virulence factor coronatine. Proc. Natl. Acad. Sci. USA 105, 7100-7105.

Kitaoka, N., Matsubara, T., Sato, M., Takahashi, K., Wakuta, S., Kawaide, H., Matsui, H., Nabeta, K., Matsuura, H., 2011. Arabidopsis CYP94B3 encode jasmonoyl-L-isoleucine 12-hydroxylase, a key enzyme in the oxidative catabolism of jasmonate. Plant Cell Physiol. 52, 1757-1765.

Koda, Y. and Okazawa, Y., 1988. Detection of potato tuber-inducing activity in potato leaves and old tubers. Plant Cell Physiol. 29, 969-974.

Koo, A.J.K., Cooke, T. F., Howe, G. A., 2011. Cytochrome P450 CYP94B3 mediates catabolism and inactivation of the plant hormone jasmonoyl-L-isoleucine. Proc. Natl. Acad. Sci. USA 108, 9298-9303.

Koo, A.J.K., Gao, X., Jones, A.D., Howe, G.A., 2009. A rapid wound signal activates the systemic synthesis of bioactive jasmonates in Arabidopsis. Plant J. 59, 974-986.

Koo, A.J.K. and Howe, G.A., 2012. Catabolism and deactivation of the lipid-derived hormone jasmonoyl-isoleucine. Front. Plant Sci. 3, 19.

Matsuura, H., Takeishi, S., Kitaoka, N., Sato, C., Sueda, K., Masuta, C., Nabeta, K., 2012. Transportation of de novo synthesized jasmonoyl isoleucine in tomato. Phytochemistry 83, 25-33.

Miersch, O., Neumerkel, J., Dippe, M., Stenzel, I., Wasternack, C., 2008. Hydroxylated jasmonates are commonly occurring metabolites of jasmonic acid and contribute to a partial switch-off in jasmonate signaling. New Phytol. 177, 114-127.

Sato, C., Aikawa, K., Sugiyama, S., Nabeta, K., Masuta, C., Matsuura, H., 2011. Distal transport of exogenously applied jasmonoyl-isoleucine with wounding stress. Plant Cell

Physiol. 52, 509-517.

Schaller, A. and Stintzi, A., 2009. Enzymes in jasmonate biosynthesis -Structure, function, regulation. Phytochemistry 70, 1532-1538.

Seto, Y., Hamada, S., Matsuura, H., Matsusige, M., Satou, C., Takahashi, K., Masuta, C., Matsui, H., Nabeta, K., 2009. Purification and cDNA cloning of a wound inducible glucosyltransferase active toward 12-hydroxyjasmonic acid. Phytochemistry 70, 370-379.

Staswick, P.E. and Tiryaki, I., 2004. The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in *Arabidopsis*. Plant Cell 16, 2117-2127.

Staswick, P.E., 2009. The tryptophan conjugates of jasmonic and indole-3-acetic acids are endogenous auxin inhibitors. Plant Physiol. 150, 1310-1321.

Suza, W.P., Rowe, M.L., Hamberg, M., Staswick, P.E., 2010. A tomato enzyme synthesizes (+)-7-*iso*-jasmonoyl-L-isoleucine in wounded leaves. Planta 231, 717-728.

Suza, W.P. and Staswick, P.E., 2008. The role of JAR1 in jasmonoyl-L-isoleucine production in Arabidopsis wound response. Planta 227, 1221-1232.

Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., Nomura, K., He, S.Y., Howe, G.A., Browse, J., 2007. JAZ repressor proteins are targets of the SCF^{COI1} complex during jasmonate signalling. Nature 448, 661-665.

Woldemariam, M.G., Onkokesung, N., Baldwin, I.T., Galis, I., 2012. Jasmonoyl-L-isoleucine hydrolase 1 (JIH1) regulates jasmonoyl-L-isoleucine levels and attenuates plant defenses against herbivores. Plant J. 72, 758-767.

Wasternack, C. and Kombrinka E., 2010. Jasmonates: structural requirements for lipid-derived signals active in plant stress responses and development. ACS Chem. Biol. 5, 63-77.

Yan, J., Zhang, C., Gu, M., Bai, Z., Zhang, W., Qi, T., Cheng, Z., Peng, W., Luo, H., Nan, F., Wang, Z., Xie, D., 2009. The *Arabidiopsis* CORONATINE INSENSIVE1 protein is a jasmonate receptor. Plant Cell 21, 2220-2236.

Yoshihara, T., Omer, E.A., Koshino, H., Sakamura, S., Kikuta, Y., Koda, Y., 1989. Structure of a tuber-inducing stimulus from potato leaves (*Solanum tuberosum* L). Agric. Biol. Chem. 53, 2835-2837.

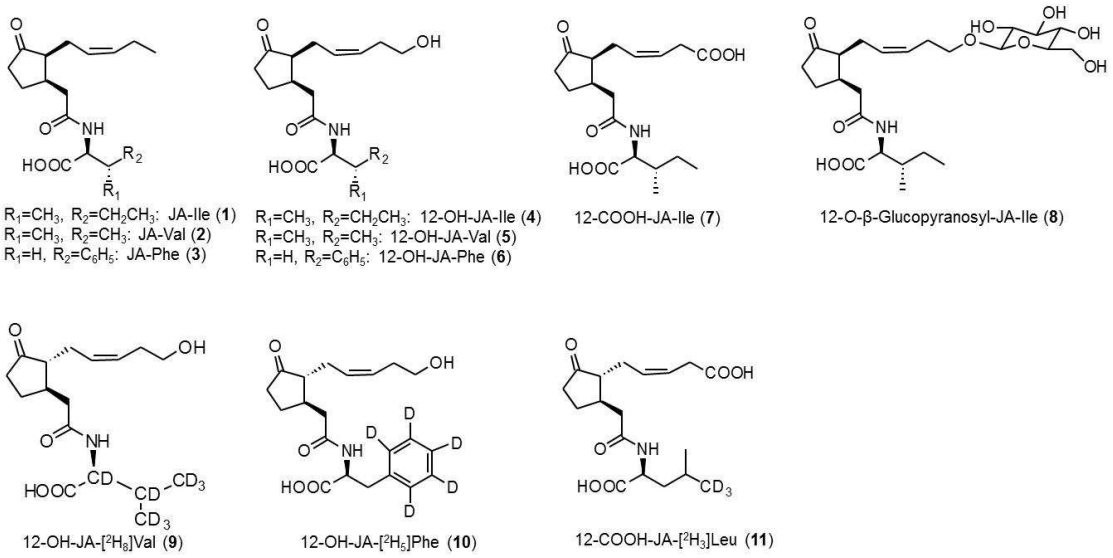


Fig. 1. Chemical structures of the jasmonates and the isotope-labeled compounds that were used or analyzed in this study.

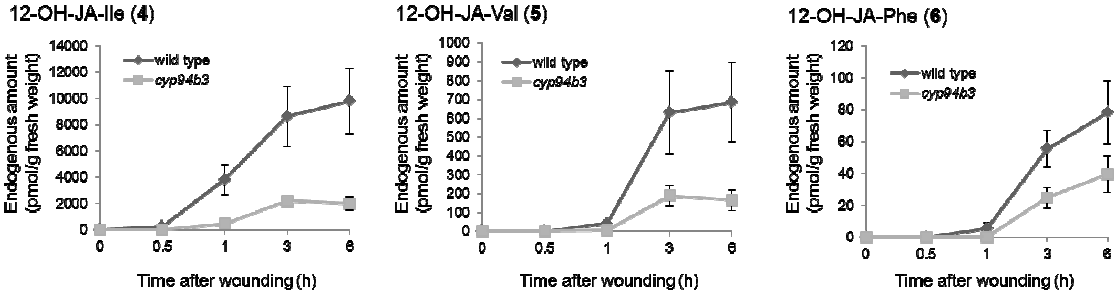


Fig. 2. Kinetics of the wound-induced accumulation of 12-OH-JA-Ile (**4**), 12-OH-JA-Val (**5**), and 12-OH-JA-Phe (**6**) in wounded leaves of wild-type and T-DNA insertion knockout (*cyp94b3*) plants.

Fully expanded younger leaves were mechanically damaged. The damaged leaves of wild-type and *cyp94b3 A. thaliana* plants were harvested at the indicated times after wounding. The wound-induced accumulation of 12-OH-JA-Ile (**4**, *Left*), 12-OH-JA-Val (**5**, *Middle*), and 12-OH-JA-Phe (**6**, *Right*) was analyzed by UHPLC MS/MS. The data are presented as the mean ±SD of five biological replicates.

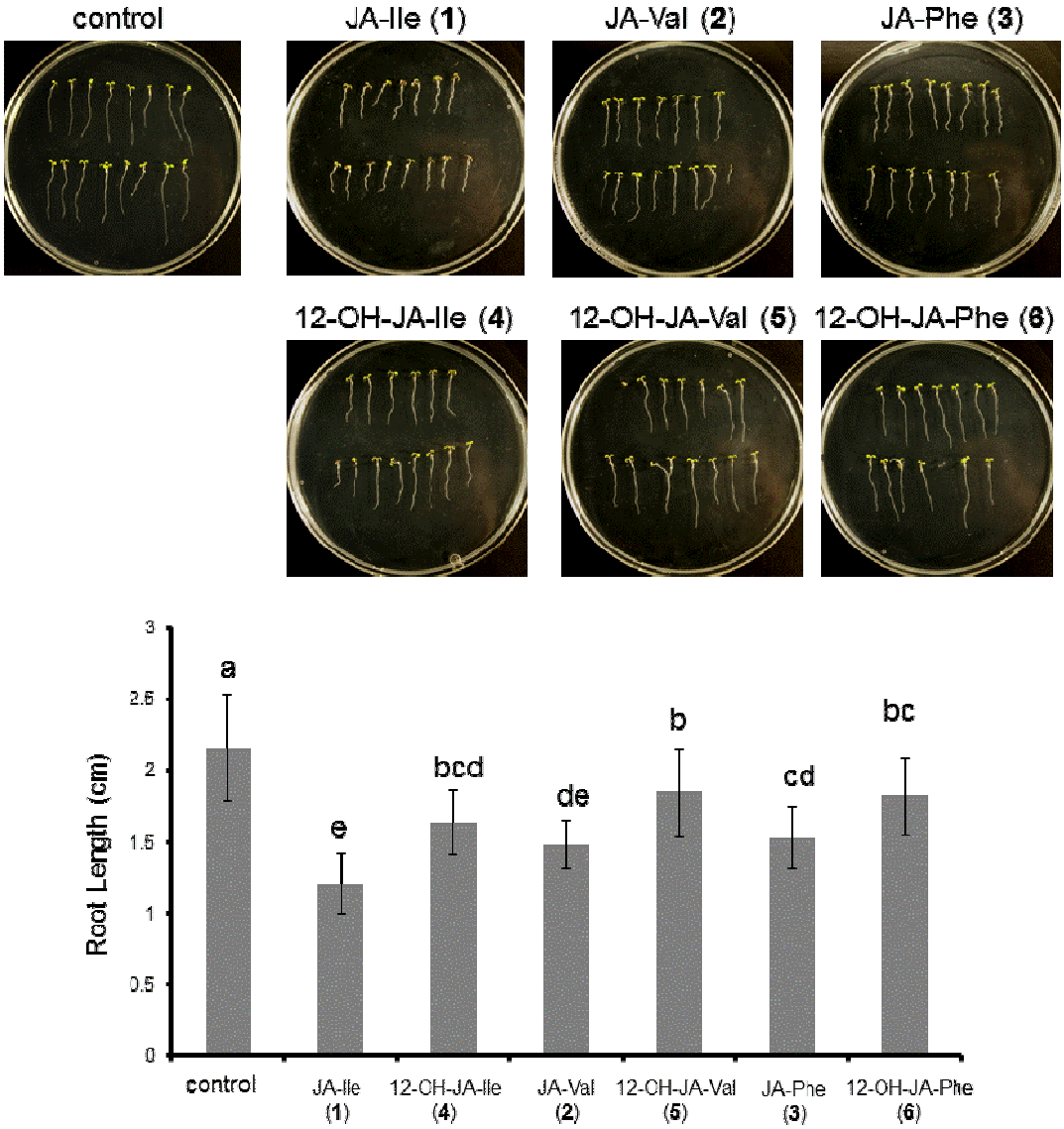


Fig. 3. Root-growth inhibition assays using 10-day-old WT *A. thaliana* seedlings grown in the presence of 50 mM JA-amino acid conjugates and 12-OH-JA-amino acid conjugates.

The bars indicate mean the root length of 25 seedlings with the SD. Means without a common letter differ, $p < 0.01$ (Tukey-Kramer test).

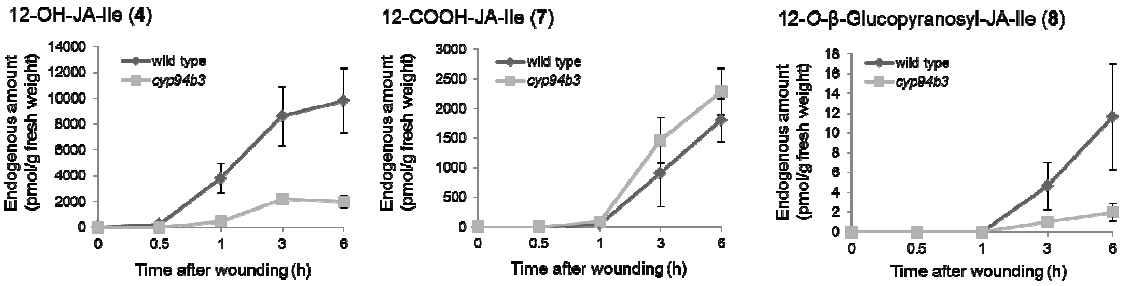
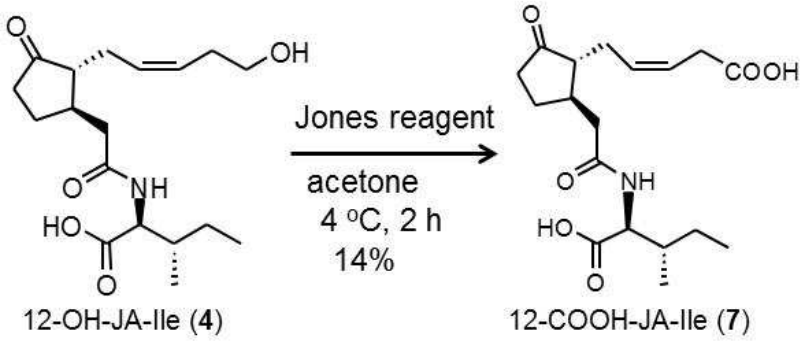
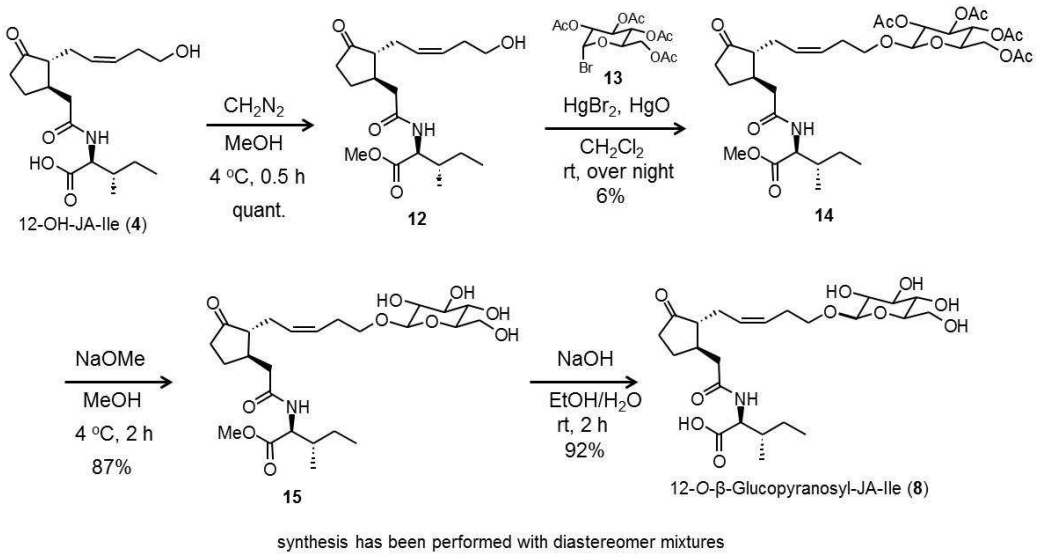


Fig. 4. Kinetics of the accumulation of jasmonates in *A. thaliana* seedlings following wounding.

Fully expanded young leaves of 5-week-old *A. thaliana* seedlings were mechanically damaged. The damaged leaves were harvested at the indicated times. The wound-induced accumulation of 12-OH-JA-Ile (**4**), 12-COOH-JA-Ile (**7**), and 12-*O*-β-glucopyranosyl-JA-Ile (**8**) was analyzed by UHPLC MS/MS. The data are presented as the mean ±SD of five biological replicates.



Scheme 1. Synthesis of 12-COOH-JA-Ile (**7**).



Scheme 2. Synthesis of 12-*O*-β-glucopyranosyl-JA-Ile (**8**)

Table 1. Substrate specificity of CYP94B3 for jasmonic acid amino acid conjugates.

	JA-Ile	JA-Leu	JA-Val	JA-Phe	JA-Ala	JA-Gly	JA-Trp	JA-ACC
CYP94B3	+	+	+	+	n.d.	n.d.	n.d.	n.d.

+, detection of oxidative product

n.d.: not detected

ACC: 1-aminocyclopropane-1-carboxylic acid

Table 2. Quantification of the endogenous amounts of 12-OH-jasmonic acid amino acid conjugates, 12-COOH-JA-Ile (**7**), and 12-*O*-β-glucopyranosyl-JA-Ile (**8**) in various higher plants after wounding.

		12-OH-JA-Ile (**4**) [a)]	12-OH-JA-Val (**5**) [a)]	12-OH-JA-Phe (**6**) [a)]	12-COOH-JA-Ile (**7**) [a)]	12-*O*-β-Glucopyranosyl-JA-Ile (**8**) [a)]
Arabidopsis thaliana	Unwounded	n.d.	n.d.	n.d.	n.d.	n.d.
	1 h	3792±1141	41±11	trace	48±29	n.d.
	3 h	8633±2293	631±221	55±12	913±574	4±2
	6 h	9813±2473	686±209	78±39	1809±361	11±5
Nicotiana tabacum	Unwounded	n.d.	n.d.	n.d.	n.d.	n.d.
	1 h	868±251	17±6	trace	6±2	n.d.
	3 h	2000±548	22±7	20±5	277±85	18±6
	6 h	1600±229	40±16	22±3	198±21	23±6
Glycine max	Unwounded	n.d.	n.d.	n.d.	n.d.	n.d.
	1 h	868±252	22±11	9±6	64±18	n.d.
	3 h	1197±547	68±6	24±4	312±67	18±5
	6 h	1599±228	92±31	37±8	433±126	22±5

a): pmol/g fresh weight, n.d.: not detected, trace: a trace peak was detected

The endogenous amounts of 12-OH-JA-Ile (**4**), 12-OH-JA-Val (**5**), 12-OH-JA-Phe (**6**), 12-COOH-JA-Ile (**7**), and 12-*O*-β-glucopyranosyl-JA-Ile (**8**) in *Arabidopsis thaliana*, *Nicotiana tabacum*, and *Glycine max* were measured using a UHPLC MS/MS system. Isotope-labeled compounds were employed as internal standards. The data are the mean ± SD (n = 5).