RESEARCH PAPER

CYP94-mediated jasmonoyl-isoleucine hormone oxidation shapes jasmonate profiles and attenuates defence responses to Botrytis cinerea infection

Yann Aubert1,*, Emilie Widemann1,2,*, Laurence Miesch3, Franck Pinot2 and Thierry Heitz1,†

1 Institut de Biologie Moléculaire des Plantes, Unité Propre de Recherche 2357 du Centre National de la Recherche Scientifique, Université de Strasbourg, 12 rue du Général Zimmer 67084 Strasbourg Cedex, France
2 Institut de Biologie Moléculaire des Plantes, Unité Propre de Recherche 2357 du Centre National de la Recherche Scientifique, Université de Strasbourg, 28 rue Goethe, 67083 Strasbourg Cedex, France
3 Laboratoire de Chimie Organique Synthétique, Institut de Chimie, Unité Mixte de Recherche 7177 Université de Strasbourg—Centre National de la Recherche Scientifique, 1 rue Blaise Pascal 67008 Strasbourg Cedex, France
* These authors contributed equally to this work.
† To whom correspondence should be addressed. E-mail: thierry.heitz@ibmp-cnrs.unistra.fr

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Abstract

Induced resistance to the necrotrophic pathogen Botrytis cinerea depends on jasmonate metabolism and signalling in Arabidopsis. We have presented here extensive jasmonate profiling in this pathosystem and investigated the impact of the recently reported jasmonoyl-isoleucine (JA-Ile) catabolic pathway mediated by cytochrome P450 (CYP94) enzymes. Using a series of mutant and overexpressing (OE) plant lines, we showed that CYP94B3 and CYP94C1 are integral components of the fungus-induced jasmonate metabolic pathway and control the abundance of oxidized conjugated but also some unconjugated derivatives, such as sulfated 12-HSO4-JA. Despite causing JA-Ile overaccumulation due to impaired oxidation, CYP94 deficiency had negligible impacts on resistance, associated with enhanced JAZ repressor transcript levels. In contrast, plants overexpressing (OE) CYP94B3 or CYP94C1 were enriched in 12-OH-JA-Ile or 12-COOH-JA-Ile respectively. This shift towards oxidized JA-Ile derivatives was concomitant with strongly impaired defence gene induction and reduced disease resistance. CYP94B3-OE, but unexpectedly not CYP94C1-OE, plants displayed reduced JA-Ile levels compared with the wild type, suggesting that increased susceptibility in CYP94C1-OE plants may result from changes in the hormone oxidation ratio rather than absolute changes in JA-Ile levels. Consistently, while feeding JA-Ile to seedlings triggered strong induction of JA pathway genes, induction was largely reduced or abolished after feeding with the CYP94 products 12-OH-JA-Ile and 12-COOH-JA-Ile, respectively. This trend paralleled in vitro pull-down assays where 12-COOH-JA-Ile was unable to promote COI1–JAZ9 co-receptor assembly. Our results highlight the dual function of CYP94B3/C1 in antimicrobial defence: by controlling hormone oxidation status for signal attenuation, these enzymes also define JA-Ile as a metabolic hub directing jasmonate profile complexity.

Key words: Antifungal defence, Arabidopsis, cytochrome P450, hormone homeostasis, jasmonate catabolism, signalling.

Introduction

Interaction of plants with pathogenic micro-organisms initiates cascades of molecular events in the host that are rapidly transduced into specific changes in hormone accumulation and distribution. Upon pathogen attack, typical hormones

Abbreviations: ANOVA, analysis of variance; dpi, days post-inoculation; ET, ethylene; FW, fresh weight; JA, jasmonic acid; JA-Ile, jasmonoyl-isoleucine; KO, knockout; OE, overexpressing; SA, salicylic acid; UPLC-MS/MS, ultra-performance liquid chromatography coupled to tandem mass spectrometry; WT, wild type.

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associated with induced defence such as salicylic acid (SA), ethylene (ET), and jasmonic acid (JA) undergo an increase in their abundance, due to stimulation of their biosynthetic pathways (De Vos et al., 2005). In addition, other hormones such as auxin, gibberellins, abscisic acid, cytokinins, and brassinosteroids, generally linked to plant growth or development, also modulate the plant innate immune system. Pathogen attacks reconfigure plant hormonal networks where positive or negative cross-talks are optimized to induce appropriate defence responses leading to elevated resistance (Hoffmann et al., 2011; Robert-Seilaniantz et al., 2011; Pieterse et al., 2012). For example, one way that SA suppresses JA responses is by reducing levels of the transcription factor ORA59, which targets GCC boxes in some JA-responsive promoters (Van der Does et al., 2013). In addition to cross-talk, plants have evolved complex mechanisms to tightly control the timing and amplitude of individual hormonal pulses by the proper balance of biosynthesis and turnover dynamics.

The JA pathway has been studied extensively in terms of biosynthesis and signalling, mostly in defence-related but also in developmental processes (Browse, 2009; Fonseca et al., 2009; Wasternack and Hause, 2013).

JA, a cyclopentanone fatty acid derivative, can be converted into a plethora of related compounds (collectively referred to as jasmonates, JAs) by oxidation, conjugation to amino acids or sugars, or other modifications (Koo and Howe, 2012; Wasternack and Hause, 2013). The co-ordinated accumulation of diverse jasmonates into a particular blend due to the action of specialized enzymes constitutes the ‘jasmonate signature’ of a given tissue. The full complement of genes/enzymes leading to this metabolic complexity, their regulation, and their biological functions still need to be elucidated.

JA signalling is a prominent example of a plant-specific paradigm of hormone signalling that proceeds through target gene derepression (Santner and Estelle, 2009). JA is a pro-hormone that acquires biological activity through Jasmonate Resistant 1 (JAR1)-catalysed conjugation towards (+)-7-iso-jasmonoyl-isoleucine (JA-Ile). JA-Ile directs the assembly of a co-receptor composed of the CORONATINE INSENSITIVE1 (COI1) F-box protein and JASMONATE ZIM-DOMAIN (JAZ) proteins (Chini et al., 2007; Thines et al., 2007; Sheard et al., 2010). Arabidopsis possesses 12 JAZ genes (Chung et al., 2008; Pauwels and Goossens, 2011) encoding repressors that, at a low JA-Ile concentration, block the action of transcription factors at promoters of JA-responsive genes. In conditions that stimulate jasmonate biosynthesis such as biotic stress, JAZ proteins engaged in the JA-Ile-promoted co-receptor are ubiquitinated by the SCF\(^{COI1}\) ubiquitin E3 ligase before their proteolytic elimination by the 26S proteasome, relieving the repression of target gene transcription. As JAZ genes are also JA-Ile responsive and stress responsive, the regulatory circuit also encodes its rapid resetting to the repressed state to prevent excessive resource allocation to defence.

In addition to the action of JAZ and other repressors on target gene promoters (Nakata et al., 2013), attenuation of JA signalling also operates at the metabolic level by modification of the hormonal signal. We and others have identified members of the cytochrome P450 family (CYP94) as major actors in the catabolitic turnover of the hormone during the leaf wound response (Kitaoka et al., 2011; Koo et al., 2011; Heitz et al., 2012). CYP94B3 and CYP94C1 \(\omega\)-oxidize JA-Ile at the C12 position, leading to the sequential accumulation of 12-OH-JA-Ile and 12-COOH-JA-Ile (Heitz et al., 2012). As with other plant hormones (Mizutani and Ohta, 2010), there is some evidence that JA-Ile oxidation corresponds to hormonal inactivation, as plants can be rendered JA insensitive by overexpressing catabolic enzymes (Koo et al., 2011; Heitz et al., 2012). We recently described the existence of an additional JA-Ile turnover pathway in Arabidopsis wounded leaves, through the amidohydrolases IAR3 and ILL6, which cleave the JA-Ile conjugate, and also define an indirect route generating tuberonic acid (12-OH-JA). These interconnected pathways define a new metabolic grid with strong implications for jasmonate homeostasis (Widemann et al., 2013).

The requirement of the JA pathway for defence against necrotrophic pathogens has been detected in various pathosystems (Penninckx et al., 1998; Vijayan et al., 1998). Infection by the broad-spectrum fungal pathogen Botrytis cinerea induces large-scale sequential changes in Arabidopsis genome expression that have recently been analysed with high temporal resolution (Windram et al., 2012). Mutants impaired in the core JA biosynthetic pathway such as allene oxide synthase (aos) or 12-oxophytodienoic acid reductase (Niekst et al., 2004; Chehab et al., 2011), or in hormone perception (Thomma et al., 1998), do not activate JA-dependent defences and are generally susceptible to this fungus. An opr3 Arabidopsis mutant was later found to be a conditional knockout (KO) that produces significant JA upon fungal infection, explaining why this line is less susceptible than aos mutant (Chehab et al., 2011). However, recent data have independently identified 12-oxophytodienoic acid-specific regulation of callose deposition in B. cinerea-infected OPR3-deficient tomato plants (Scalschi et al., 2015). B. cinerea also induces SA-dependent responses, as part of a strategy to antagonize JA-Ile-mediated resistance (El Oirdi et al., 2011). It is known that distinct JA-Ile hormonal networks are operating to control inducible leaf defence against different types of attackers and are integrated by separate sets of transcription factors. MYC2 integrates JA/abscisic acid signals and defines a wound/insect-specific branch that can be probed with vegetative storage protein (VSP) marker induction; ERF1/ORAA5 integrate JA/ET signals in a pathogen-triggered branch that is monitored by PDF1.2 or PR4 expression ( Lorenzo et al., 2004; Pre et al., 2008). Both branches work antagonistically (Dombrecht et al., 2007), leading to functional prioritization in subgroups of JA-Ile-regulated defences and associated resistance against microbes or insects (Lorenzo et al., 2004; Nickstadt et al., 2004; Verhage et al., 2011).

Although many genetic factors leading to B. cinerea resistance are known, the way plants regulate JA-Ile homeostasis in necrotrophic pathosystems is poorly understood. For example, the diversity, abundance, and metabolic relationships of JAs accumulated in response to B. cinerea have not
been examined in detail. The specific aim of this work was to evaluate the contribution and impact of the CYP94 catabolic pathway on microbial pathogen defence. We established the complex jasmonate profile triggered by the sequenced B05.10 strain of *B. cinerea* and showed that activation of the CYP94B3/CYP94C1-mediated JA-Ile catabolic pathway contributes to draw a unique pattern of oxidized conjugated and unconjugated jasmonates. Blocking or enhancing genetically JA-Ile oxidation respectively depleted or boosted the accumulation of the 12-OH-JA-Ile and 12-COOH-JA-Ile derivatives, but also affected the abundance of unconjugated JA derivatives. The CYP94-misexpressing lines showed a number of gene deregulations along the JA signalling and defence pathway, illustrating the role of JA-Ile oxidation in optimal antimicrobial output. Interestingly, in CYP94C1-overexpressing (C1-OE) plants, impaired defence induction and increased susceptibility to infection was observed while JA-Ile was maintained near wild-type (WT) levels, suggesting that, in this genotype, JA-Ile signalling may be non-functional due to excessive hormone oxidation.

**Materials and methods**

**Plant growth and methods**

The *Arabidopsis thaliana* genotypes used were all in the Col-0 ecotype and were grown under a 12 h light/12 h dark photoperiod in a growth chamber. The following T-DNA insertion lines were obtained from the Nottingham Arabidopsis Stock Center (NASC): cyp94b1-1 (SALK_129672), cyp94b2-1 (CS302217), and cyp94c1-1 (SALK_55455). The double cyp94b1c3c1 mutant was obtained as described by Heitz et al. (2012). The triple cyp94b1b3c1 mutant was obtained after crossing the double mutant with cyp94b1-1 and PCR genotyping of F2 progeny. The line overexpressing CYP94C1 (C1-OE-3B) was described previously by Heitz et al. (2012). The line overexpressing CYP94B3 (B3-OE) was obtained from Dr Yves Millet (Massachusetts General Hospital, Boston, USA).

For fungal infection experiments, 6-week-old plants were used. *B. cinerea* strain B05.10 was used to inoculate four to seven leaves of *N. benthamiana* leaf sections. For seedling jasmonate treatment, surface-sterilized seeds were germinated in synthetic media for 5 d at 22°C in the following medium: 1× Murashige and Skoog liquid medium, supplemented with 0.5% sucrose and 0.05% MES buffer. Seedlings were grown for 7 d before gentle addition of 30 µM of synthetic JA-Ile, 12-OH-JA-Ile, or 12-COOH-JA-Ile obtained as described by Widemann et al (2013). At increasing time points after treatment, seedlings were quickly harvested and flash frozen in liquid nitrogen before storing at –80°C until RNA extraction.

**B. cinerea relative quantification**

*B. cinerea* growth in inoculated plants was determined by quantification of fungal DNA relative to plant DNA. For plant/fungal DNA extraction, 400 µl of buffer (0.2 M Tris-/HCl, pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added to frozen leaf powder [50–70 mg of fresh weight (FW)] from infected plants. The material was ground for 30 s with a Precellys 24 tissue homogenizer (Bertin Technologies, Montigny-Le-Bretonneux, France). The homogenate was cleared by centrifugation. DNA in the supernatant was precipitated with isopropanol, and the DNA was finally resuspended in 10 mM Tris/HCl, pH 8. Measurement of colonization of leaves by *B. cinerea* was based on real-time PCR quantification of the fungal CUTINASE signal (Z69264) relative to that of *Arabidopsis ACTIN2* measured as described by Berr et al. (2010). Primer sequences are detailed in Supplementary Table S1 available at JXB online.

**Quantitative reverse transcription (RT)-PCR gene expression assays**

Total RNA was extracted from plant leaves with TRIzol reagent (Molecular Research Center). One microgram of RNA was reverse transcribed using the ImProm-II reverse transcription system (Promega, Madison, WI). Real-time PCR was performed on 10 ng of cDNA as described by Berr et al. (2010) using a LightCycler 480 II instrument (Roche Applied Science). The housekeeping genes *EXP* (At1g53770), *TIP41* (At4g34720), and *GAPDH* (At1g13440) were used as internal references for rosette-stage plants and *TIP41* and *GAPDH* for experiments with seedlings. Gene-specific primer sequences used for quantitative RT-PCR are listed in Supplementary Table S1.

**COI1 and JAZ9 recombinant protein expression**

COI1 protein was transiently expressed in *Nicotiana benthamiana* leaves. To this end, the COI1 open reading frame (At2g39940) was cloned in the *Sall* and *Not1* sites of pENTR1a, before recombining into the pGWB20 plasmid, introducing a C-terminal 10× myc tag. The construct was transformed into *Agrobacterium tumefaciens* GV3101 strain, which was infiltrated into *N. benthamiana* leaf sectors. After 3–5 d, leaf material was harvested and stored at –80°C until used for expression as a tagged COI1 protein source for pull-down assay. JAZ9 cDNA was amplified and cloned in the *BamHI* and *Not1* sites of pENTR1a plasmid, before recombining into pHMGWA plasmid, creating a His-maltose binding protein–JAZ9–His fusion protein. The construct was introduced into *Escherichia coli* BL21 strain. For protein purification, resuspended induced bacteria pellet was sonicated in lysis buffer (20 mM Tris/HCl, pH 8, 300 mM NaCl, 5% glycerol, 1 mM EDTA, 0.1% Tween 20, 1 mM PMSF, and 1 mg ml–1 of lysozyme) on ice. The lysate was centrifuged and the supernatant was filtered (0.22 µm) before adding imidazole to 50 mM. The fusion protein was purified on a HisTrap column (GE Healthcare) with an ATKA Purifier 10 (GE Healthcare) chromatographic system. Imidazole was used at increasing concentrations for successive column loading (50 mM), washing (100 mM), and elution (350 mM). The imidazole was eliminated from purified protein fractions by gel filtration.

**Pull-down assay**

Protein extraction and pull-down conditions were adapted from Thines et al. (2007). Soluble *N. benthamiana* leaf protein extract was prepared by manually grinding 1 g of frozen powder in extraction buffer (50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 25 mM imidazole, 10% glycerol, 0.1% Tween 20, 20 mM β-mercaptoethanol, Complete Mini Protease Inhibitor tablet EDTA-free (Roche), and 10 µM MG132). The supernatant obtained after centrifugation contained COI1–myc and was used for pull-down assays. The concentration of total protein in the extract was evaluated by the Bradford method and a BSA range.

Pull-down assays were conducted in COI1 extraction buffer in a total volume of 400 µl containing 600 µg of the total 35S::COI1–myc leaf extract and 60 µg of purified JAZ9 fusion protein. Reactions were incubated for 30 min at 4°C by gentle shaking in the absence or presence of jasmonate conjugates. One hundred microliters of...
Ni-NTA agarose beads were then added and the mixtures were incubated for 15 min at 4 °C. After three washings with binding buffer, the beads were resuspended in elution buffer (binding buffer supplemented with 300 mM imidazole). Eluates were analysed by Western blotting with anti-myc primary antibody.

**Metabolite profiling**

Jasmonates and SA were identified and quantified in plant extracts by ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) as described by Heitz et al. (2012) and Widemann et al. (2013), except that the extraction solvent also contained gentisic acid as an internal standard for SA determination. SA was detected in methanol extracts with the mass transition 137>93 in negative mode. The relative quantification in samples was achieved by reporting MS peak areas relative to internal standard peak area and mass of biological material. Absolute quantifications of jasmonates were determined by comparison of the sample signal with dose–response curves established with pure compounds. Chemical synthesis of 12-OH-JA-Ile and 12-COOH-JA-Ile from commercial methyl jasmonate has been described by Widemann et al. (2013).

**Statistical analysis**

All statistical analysis was performed using GraphPad Prism 5. Comparisons of sample means were performed by one-way analysis of variance (one-way ANOVA) and Bonferroni’s post-hoc multiple comparisons tests (P<0.05 for both) to determine whether sample means were significantly different.

**Results**

**B. cinerea infection triggers a complex jasmonate profile**

We established recently that plants react to leaf wounding by accumulating a blend of conjugated and/or oxidized jasmonates (Heitz et al., 2012; Widemann et al., 2013). In contrast to wounding, jasmonate analysis in the Arabidopsis response to fungi such as B. cinerea has generally been limited to JA quantification (Yang et al., 2007; La Camera et al., 2011). In particular, no information is available concerning the accumulation of the JA-Ile hormone catabolites generated by cytochrome P450 of the CYP94 family (Heitz et al., 2012).

WT plants were drop inoculated with fungal spores to generate two infection sites per leaf. This set-up generated enough strongly stimulated material for metabolic analysis or gene expression and allowed us to monitor plant resistance on individual lesions. Leaf extracts were prepared from infected plants and submitted to UPLC-MS/MS analysis. The spectrum of analysed jasmonates included JA, 12-OH-JA, and its glycosylated (12-O-Glc-JA) or sulfated (12-HSO₄-JA) derivatives, which occur abundantly in different plant species (Gidda et al., 2003; Miersch et al., 2008), the hormone JA-Ile, and its two oxidation products 12-OH-JA-Ile and 12-COOH-JA-Ile (for compound structure, see Fig. 7). All JAs were close to, or below, the detection limit in leaves from uninoculated plants or even 1 d post-inoculation (dpi). Analysis was thus focused on profiling leaf extracts at 2 and 3 dpi when disease symptoms were visible. The data are presented in Fig. 1. All JAs analysed were found to accumulate at different levels upon infection. JA and its hydroxylated derivative 12-OH-JA, also known as tuberonic acid (Miersch et al., 2008), were the most abundant and built up to 6–7 nmol g⁻¹ of FW at 3 dpi (Fig. 1A, C). 12-OH-JA can also be further modified to 12OH-JA glucoside and 12OH-JA sulfate (Gidda et al., 2003; Miersch et al., 2008; Widemann et al., 2013), and these compounds were readily detected in infected leaves (Fig. 1E, G).

JA-Ile hormone showed maximal levels around 80–100 pmol g⁻¹ of FW at 3 dpi (Fig. 1B), which is one order of magnitude lower than peak accumulation at 1 h post-wounding (Heitz et al., 2012; Widemann et al., 2013). Finally, the JA-Ile catabolites 12-OH-JA-Ile and 12-COOH-JA-Ile were quantified at 30–90 and 600 pmol g⁻¹ of FW, respectively, at 3 dpi (Fig 1D, F). Collectively, these data extend the complexity of the jasmonate profile triggered in response to B. cinerea infection, and set the framework to study the impact of JA-Ile oxidation on jasmonate homeostasis.

**Manipulating CYP94 expression impacts the jasmonate profile upon B. cinerea infection**

JA-Ile oxidized derivatives are formed upon mechanical wounding due to the transcriptional upregulation of two CYP94 genes, CYP94B3 and CYP94C1 (Kitaoka et al., 2011; Koo et al., 2011; Heitz et al., 2012). The accumulation of these oxidation products after fungal infection prompted us to investigate the expression of the six CYP94 genes in this pathosystem. CYP94D1 presents features of a pseudogene (Y. Aubert, unpublished data) and its transcripts were undetectable in leaves. CYP94B2 and CYP94D2 showed weak or moderate expression, respectively, in control leaves, which declined in diseased leaves (Fig 2A). Three genes, CYP94B1, CYP94B3, and CYP94C1 were clearly upregulated, with CYP94B3 showing the highest fold change compared with unstimulated leaves (Fig. 2A, T0 set to 1 for each gene), due to its very low basal expression level. When data were calculated as expression levels (without fixing the T0 value, with the target signal corrected by the signal of two reference genes), due to its very low basal expression level. When data were calculated as expression levels (without fixing the T0 value, with the target signal corrected by the signal of two reference genes), CYP94C1 appeared as the most expressed member, peaking at 2 dpi, followed by CYP94B3, and then by the much lower expression of CYP94B1 (Fig. 2B). The expression dynamics of the CYP94B3 and CYP94C1 genes were therefore most consistent with a role in the formation of oxidized JA-Ile conjugates in infected tissues. We also determined that the second pathway for JA-Ile turnover in wounded leaves (Widemann et al., 2013), involving the amidohydrolases IAR3 and ILL6, was also upregulated in infected leaves, with IAR3 showing the strongest response (Supplementary Fig. S1 available at JXB online).

We next used a set of single, double, and triple mutants in CYP94B1, CYP94B3, and CYP94C1 genes (Heitz et al., 2012) to dissect their contribution to JA-Ile oxidation and more generally to JA homeostasis upon B. cinerea infection. The seven plant genotypes were the previously described single cyp94b3 and cyp94c1, and double cyp94b3c1 mutants, and a line ectopically overexpressing CYP94C1 (C1-OE) under the cauliflower mosaic virus 35S promoter (Heitz et al., 2012), along with the newly generated CYP94B3
Fig. 1. Jasmonate profiles and SA levels in CYP94 loss- or gain-of-function mutants in response to B. cinerea. Six-week-old plants were drop inoculated with a suspension containing 2.5 × 10⁶ spores ml⁻¹, leaves were harvested at 2 or 3 dpi, and jasmonates extracted and quantified by UPLC-MS/MS. (A) JA; (B) JA-Ile; (C) 12-OH-JA; (D) 12-OH-JA-Ile; (E) 12-O-Glc-JA; (F) 12-COOH-JA-Ile; (G) 12-HSO₄⁻-JA; (H) SA. Mutant genotypes are indicated as follows: cyp94b3, b3; cyp94c1, c1; cyp94b3c1, b3c1; cyp94b1b3c1, b1b3c1; CYP94B3 overexpressor, B3-OE; CYP94C1 overexpressor, C1-OE. Histograms represent the means±SEM of six biological replicates from two independent experiments (compounds in A–D) for the block of five genotypes, or from one experiment with three biological replicates for the block of three genotypes (all compounds). Statistical analysis was applied separately at 2 or 3 dpi to blocks of five and three genotypes, each comprising a separate WT control (WT1 or WT2). Columns labelled with different letters indicate a significant difference between genotypes from a given block as determined by one-way ANOVA and Bonferroni post-tests (P<0.05).
overexpressor (B3-OE) or cyp94b1b3c1 triple mutant. The expression characteristics of these latter lines are described in Supplementary Fig. S2 available at JXB online. Infected leaves were collected at 2 and 3 dpi, methanol extracts were submitted to UPLC-MS jasmonate profiling, and the abundance of JAs was compared among genotypes as presented in Fig. 1. Because of the large number of plant populations handled in the study, we treated them in parallel as two separate sets of plants each including a WT control population (WT1 and WT2). Mutants impaired in JA-Ile oxidase activity were expected primarily to display changes in JA-Ile and related conjugates; however, pathway interruption redirects flux to more distant compounds, as reported previously (Heitz et al., 2012). The levels of JA, the most upstream pathway compound analysed (Fig. 1A), were enhanced in cyp94 mutants and were reduced by 30–40% in both OE lines at 3 dpi, consistent with altered JA consumption in these lines. JA-Ile, the endogenous CYP94 substrate, was also differentially affected by CYP94 mis-expression: its levels were increased as expected in most KO lines at 2 and 3 dpi, and were reduced by about 50% in B3-OE, in accordance with enhanced hormone turnover. In contrast, JA-Ile content appeared similar to that of the WT in the C1-OE line, as established in two independent experiments (Fig. 1B). The accumulation of JA-Ile oxidation products was found to be largely affected by CYP94 depletion or overexpression (Fig. 1D, F). 12-OH-JA-Ile, the major in vitro product of CYP94B3 activity (Heitz et al., 2012) but the least abundant compound analysed here, was reduced in the double and triple mutant lines (Fig. 1D, 2 dpi) compared with the WT, and was enhanced 6–15-fold by CYP94B3 overexpression, or 5-fold by CYP94C1 overexpression (Fig. 1D). In addition, levels of 12-COOH-JA-Ile, the major in vitro product of CYP94C1 activity (Heitz et al., 2012), were nearly undetectable in all cyp94c1-deficient lines (Fig. 1F), and were boosted 15-fold over the WT in the C1-OE line at 3 dpi. These data demonstrated the primary role of CYP94B3 and CYP94C1 in the accumulation of oxidized derivatives of the JA-Ile hormone in response to B. cinerea infection. We showed previously that, upon leaf wounding, 12-O-Glc-JA and 12-HSO3-JA derive from 12-OH-JA (tuberonic acid) generated through 12-OH-JA-Ile cleavage by the amidohydrolases IAR3 and ILL6 (Widemann et al., 2013). In response to fungal infection, 12-OH-JA levels were only weakly affected in single or multiple cyp94 mutants, but were decreased in OE lines, most significantly in B3-OE at 3 dpi (Fig. 1C). The two 12-OH-JA derivatives were accumulated after fungal infection, with much higher 12-O-Glc-JA levels in both OE lines (Fig. 1E, G), but were differentially affected by CYP94 mutations. 12-O-Glc-JA was only moderately reduced in the triple cyp94b1b3c1 mutant, whereas 12-HSO3-JA accumulation was strongly impaired in all CYP94B3-deficient lines, indicating distinct precursor trafficking for these two 12-OH-JA derivatives.

CYP94-mediated JA-Ile catabolism impacts B. cinerea resistance responses

We then examined whether these metabolic alterations, particularly those related to JA-Ile levels, had an impact on B. cinerea resistance. Representative disease symptoms recorded at 3 dpi on the different genotypes are shown in Fig. 3A. Lesion diameters were scored as shown in Fig. 3B, and their size distribution was established for all genotypes (Fig. 3C). Average lesion sizes were not significantly different from the WT in mutant plants, but B3-OE and C1-OE plant lines displayed much larger lesions, characteristic of a reduced resistance phenotype (Fig. 3A, B). Both OE lines displayed more large (>7.5 mm) lesions (Fig. 3C). To determine whether changes in lesion size were due to altered fungal growth, we monitored fungal DNA content in leaves by real-time PCR by amplifying a B. cinerea genomic cutinase fragment. Consistent with symptom severity, fungal DNA was 2.5-fold more abundant in the C1-OE line at 3 dpi (Fig. 3D). In contrast, the fungal DNA content was found to be increased, but not significantly, in the B3-OE line, but reduced in the triple mutant. Collectively, these data indicated that CYP94C1 and CYP94B3 expression positively correlated with stronger disease symptoms and/or growth of B. cinerea.

CYP94-mediated JA-Ile catabolism impacts defence responses to B. cinerea

Because of changes in JA profiles and resistance levels to B. cinerea infection, we investigated CYP94-modified plants for the transcriptional behaviour of marker genes in the defence cascade. PDF1.2 and PR4 encode antifungal proteins and probe the activation of the antimicrobial branch of the JA pathway (Pieterse et al., 2012; Wasternack and Hause,
Expression of both genes was readily increased in response to *B. cinerea*, with a slight enhancement in *cyp94* mutants at 2 dpi compared with the WT (Fig. 4A, C). In contrast, both *PDF1.2* and *PR4* expression was strongly impaired in OE lines at both collection times. In particular, at 2 dpi, *PDF1.2* expression was barely detectable in these lines. *B. cinerea* is countered by the induction of ET/JA-dependent defence responses (Thomma et al., 1998), but this fungus also induces to a lower extent the SA-dependent pathway, known to be more potent in defending *Arabidopsis* against biotrophic pathogens (Pieterse et al., 2012). The SA-marker *PR1* was more induced in OE lines, mostly in C1-OE (Fig. 4B), a response that was correlated with higher free SA levels (Fig. 1H), suggesting that excessive JA-Ile oxidation relieves the SA pathway that is restrained in the WT.

*ERF1* encodes a transcription factor positively regulating *PDF1.2* and *PR4* expression (Lorenzo et al., 2004). We investigated whether defence gene misregulation was associated with perturbations in *ERF1* expression. *ERF1* early expression (2 dpi) was enhanced in all *CYP94*-deficient lines (Fig. 4D), and dropped in single mutants at 3 dpi. Conversely, *ERF1* transcripts were reduced in both OE lines: C1-OE at 2 dpi and B3-OE at 3 dpi. These results showed that *CYP94B3/C1*-encoded JA-Ile oxidation transiently distorts *ERF1* expression, which only partially correlates with changes in *PDF1.2* and *PR4* expression.

In an attempt to understand why increased JA-Ile levels and *ERF1* expression did not result in enhanced defence gene expression in *cyp94* KO lines, we evaluated the expression of *JAZ* genes as signalling components repressing the JA response (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007). JA-Ile signalling and gene derepression relies primarily on pre-existing JAZ protein degradation, but strong transcriptional JAZ gene upregulation is believed to restore
target gene repression during the signal attenuation phase. Among the 12 Arabidopsis JAZ genes, most have been shown to be responsive to wounding (Chung et al., 2008) or to Pseudomonas syringae bacterial infection (Demianski et al., 2012). We showed here that JAZ1, -5, -6, 7, -8, -9 and -10 were also induced to various extents in WT plants by B. cinerea infection (Supplementary Fig. S3 available at JXB online). JAZ8 exhibited the strongest induction factor relative to T0, due to an extremely low basal level (Supplementary Fig. S3).

When the JAZ signal was normalized only with the reference gene signal, JAZ9 was the most expressed gene in infected leaves (Supplementary Fig. S3B). We analysed the behaviour of four of these genes in CYP94-modified lines. JAZ1 (Fig. 5A) and JAZ5 (Fig. 5B) displayed WT expression in both OE lines but showed a strong overinduction trend in mutant lines. In marked contrast, JAZ8 and JAZ9 expression was largely unaffected by cyp94 mutations, and was impaired in both OE lines. These data illustrated that individual JAZ repressor genes are differentially affected by the plant capacity to oxidize the JA-Ile hormone.

Oxidized JA-Ile derivatives are unable to induce JA-responsive gene expression

The monitoring of gene expression at different levels in the JA signalling cascade revealed that, despite moderate alterations in JA-Ile levels, CYP94-modified plants exhibited strong perturbations in the downstream output of the JA defence pathway. In particular, B3-OE and 94C1-OE plants, which both exhibited impaired B. cinerea resistance, had particular jasmonate profiles characterized by the overaccumulation of 12-OH-JA-Ile and 12-COOH-JA-Ile derivatives, respectively, that could influence defence signalling. To compare their capacity to promote in vitro JAZ–COI1 co-receptor assembly, the initial event in JA-Ile signalling, we assayed the three JA-Ile conjugates in a pull-down assay using tagged versions of COI1 and JAZ9 (the most expressed JAZ protein in response to B. cinerea). JA-Ile promoted a dose-dependent assembly of the co-receptor, while 12-OH-JA-Ile generated only a weak response at 50 µM (Fig. 6A), consistent with a previous report (Koo et al., 2011). Under these conditions, 12-COOH-JA-Ile produced no detectable signal, indicating that the most oxidized JA-Ile conjugate was devoid of activity in this assay.

To further assess individual signalling properties of JA-Ile oxidized derivatives, we tested their gene-inducing activity by external application to 7-d-old liquid-grown seedlings. This system allows a direct and continuous contact with the inducer without inflicting undesired stress, and results in high-amplitude responses. JA-Ile, 12-OH-JA-Ile, or 12-COOH-JA-Ile (30 µM) was applied and plantlets were analysed in kinetic studies for the transcriptional responses of pathway marker genes. JA-Ile readily induced the early and biphasic (peaks at 0.5–1 h and 6–12 h) expression of CYP94C1 (Fig. 6B) and JAZ5 (Fig. 6C), while PDF1.2 and VSP1 exhibited a delayed response as expected for late marker genes (Fig. 6D,
Comparatively, both oxidized compounds led to weak or no responses (scales on right of graphs in Fig. 6B–E). More precisely, 12-OH-JA-Ile provoked detectable responses, ranging from 1/50 of the JA-Ile response for the MYC2-target VSP1 to 1/5 for CYP94C1 at 12 h, while no PDF1.2 induction was detectable. In contrast, 12-COOH-JA-Ile was unable to induce any of the genes analysed. These data indicated that sequential JA-Ile oxidation by CYP94 enzymes resulted in an incremental decrease in hormonal activity.

**Discussion**

**Botrytis infection induces a complex array of jasmonate derivatives**

Most studies on hormone homeostasis and interactions in plant defence have focused on biosynthesis and signalling (reviewed by Hoffmann et al., 2011; Robert-Seilaniantz et al., 2011; Pieterse et al., 2012; Gimenez-Ibanez and Solano, 2013), but very little is known on how catabolic pathways intervene to generate appropriate hormone dynamics and govern precise dosage and duration of defence responses. Here, we investigated how the defence hormone JA-Ile, which is critical in resisting attack by *B. cinerea*, is turned over and how its oxidative catabolism conditions the JA metabolic network and defence output. The use of synthetic standards allowed the quantification of seven jasmonates, including the recently characterized JA-Ile catabolites (Fig. 7). Kinetic studies revealed quantitative and qualitative differences with profiles recorded previously in the wound response (Heitz et al., 2012), indicating that JA metabolic genes/enzymes and flux through specific biochemical steps undergo stimulus-specific regulation. Although unconjugated jasmonates were in similar nanomolar ranges in both responses, the three JA-Ile conjugates were much less abundant after infection than upon wounding, suggesting either lower synthesis or higher turnover in infected leaves. JA-Ile levels culminated in infected leaves at only about 1/15 of the level reached in wounded leaves, indicating that less JA may be conjugated towards JA-Ile after infection. A possibility is that the SA pathway, activated by *B. cinerea* but not by wounding, specifically represses the synthesis of JA-Ile conjugates. The rather severe wound stress procedure generally used results in a transient but vigorous JA-Ile pulse because of synchronous stimulation across the leaves. In contrast, growing fungal necrotic lesions inflict a continuous, asynchronous stimulation where a ring of cells at the infection front are in a given metabolic and signalling state, leading to a steady increase in metabolite abundance. The levels of 12-OH-JA-Ile were about one order of magnitude lower than those of 12-COOH-JA-Ile, meaning that 12-OH-JA-Ile is either further oxidized, consistent with predominant CYP94C1 expression, or undergoes other modifications. Upon wounding, where CYP94B3 expression dominates, both compounds accumulate at similar levels (Widemann et al., 2013). 12-OH-JA (tuberonic acid) was the second most abundant jasmonate after JA in
response to *B. cinerea*, and glucosylated and sulfated 12-OH-JA derivatives also accumulated. Overall, these data illustrate the dynamics of the complex JA profile triggered in response to *B. cinerea* infection. This JA signature is likely to be non-exhaustive; for example, some other JA–amino acid conjugates like JA–tyrosine or JA–phenylalanine also accumulate in *B. cinerea*-infected leaves (E. Widemann, unpublished data). These compounds are also potential CYP94 substrates, but they are inactive in promoting COI1–JAZ co-receptor assembly, and no signalling activity has yet been assigned to these compounds in *Arabidopsis*.

**CYP94B3 and CYP94C1 shape the jasmonate signature in response to *B. cinerea***

We have shown previously that the wound-induced accumulation of the oxidized compounds 12-OH-JA-Ile, 12-COOH-JA-Ile, 12-OH-JA, 12-OH-JA-Ile, and 12-HSO₄⁻JA is largely dependent on CYP94B3 and CYP94C1 activity on JA-Ile (Heitz *et al.*, 2012; Widemann *et al.*, 2013). Here, we established that CYP94C1 has the strongest response to fungal attack, with the weakest responding gene being CYP94B1. CYP94B1 was recently characterized as a close relative to CYP94B3 with similar catalytic properties (Koo *et al.*, 2014). The metabolic impact and phenotypes were observed predominantly in plants strongly overexpressing CYP94B1, while a single *cyp94b1* mutant behaved like the WT. The work presented here dissected the contribution of the three JA-Ile oxidases to the JA signature by using a series of seven CYP94-modified plant genotypes. For most compounds, the triple *cyp94b1b3c1* mutant did not accentuate the profiles of the double *cyp94b3c1* mutant, consistent with a quantitatively minor role of CYP94B1 in this pathosystem. As an exception to this trend, an impact of *cyp94b1* mutation could

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**Fig. 6.** Sequential JA-Ile ω-oxidization abolishes the capacity to promote co-receptor assembly and induction of jasmonate-responsive genes. (A) In vitro pull-down assay performed with purified His-tagged JAZ9 and *N. benthamiana* leaf extract expressing AtCOI1–myc, in the presence of increasing concentrations of JA-Ile, 12-OH-JA-Ile, or 12-COOH-JA-Ile as indicated. JAZ9 Coomassie blue staining on the membrane is shown on the lower panel to indicate equal recovery and loading. (B–E) Seven-day-old liquid-grown seedlings were incubated in the presence of 30 µM JA-Ile, 12-OH-JA-Ile, or 12-COOH-JA-Ile for the indicated time periods. Seedlings were flash frozen in liquid nitrogen and submitted to RNA extraction. One microgram of total RNA was reverse transcribed and expression of *CYP94C1* (B), *JAZ5* (C), *PDF1.2* (D), and *VSP1* (E) was determined by real-time PCR using gene-specific primers and normalized with *GAPDH* and *TIP41* as the reference genes. Histograms represent the means±SEM of three technical replicates. Expression is represented as fold induction relative to expression level at T0, which was set to 1 for each gene. Fold induction by JA-Ile (black circles) is shown on the left scale of each graph and fold induction of oxidized conjugates (12-OH-JA-Ile, open squares; 12-COOH-JA-Ile, open triangles) is shown on the right scale of each graph. Mock treatments induced no response and are not shown for clarity. The experiment was repeated and generated similar results.
be detected as a 40–50% loss of 12-O-Glc-JA in the triple cyp94b1b3c1 mutant compared with the WT2 control, while levels were not affected in cyp94b3c1 plants relative to the WT1 control. Therefore, the role of CYP94B1 is best visualized when it acts alone (in cyp94b3c1 plants) and where it may specifically contribute to the biosynthesis of 12-O-Glc-JA.

Several important findings can be drawn on the regulation of the Botrytis-triggered jasmonate circuitry (Fig. 7): (i) Most obviously, the direct dependence of 12-OH-JA-Ile and 12-COOH-JA-Ile accumulation on CYP94B3 and CYP94C1 expression, respectively, was evidenced with antagonistic effects in mutant and OE lines, which ranged over three orders of magnitude in the case of 12-COOH-JA-Ile in CYP94C1-modified genotypes (Fig. 1). These data identify a major role for CYP94C1 in JA-Ile oxidation upon B. cinerea infection. (ii) Consistent with a metabolic block, JA-Ile levels were enhanced moderately by cyp94 mutations relative to the WT, but, conversely, JA-Ile was only decreased (by half) in the B3-OE line. The C1-OE line used (C1-OE3B), although exhibiting reduced JA-Ile levels after wounding (Heitz et al., 2012), featured essentially WT JA-Ile levels after B. cinerea in two independent experiments. C1-OE bore larger fungal lesions than WT, which may stimulate more tissue to accumulate JA-Ile levels comparable to WT in total leaves, but which may be reduced on a per cell basis and attenuate signalling. This interpretation would also hold true for B3-OE, but one should keep in mind that B3 enzyme acts primarily on JA-Ile, while C1 enzyme readily uses 12-OH-JA-Ile as substrate (Heitz et al., 2012) and is less likely to directly affect JA-Ile levels. (iii) Examination of other JAs revealed that B. cinerea-induced 12-OH-JA and 12-O-Glc-JA accumulation is intact in cyp94b3 or cyp94c1 single or double mutants and therefore these compounds are unlikely to arise massively from the indirect oxidation-cleavage pathway via 12-OH-JA-Ile intermediate as after wounding (Widemann et al., 2013).
these compounds are formed from JA by successive oxidation and glucosylation by unknown enzymes (Fig. 7). In contrast, 12-HSO₄-JA accumulation was clearly CYP94B₃-dependent, pointing to its formation via 12-OH-JA-Ile through an unknown sequence of reactions. Unexpectedly, B₃-OE and C₁-OE plants had increased 12-O-Glc-JA and to a lesser extent 12-HSO₄-JA levels, suggesting that CYP94B₃ or CYP94C₁ overexpression creates a new biosynthetic route leading to these compounds, and that this may not proceed through free 12-OH-JA accumulation. Clearly, mapping all possible metabolic conversion routes between jasmonates and their regulation under different stress or developmental conditions needs deeper investigation, including the characterization of new enzyme activities.

Perturbed JA-Ile turnover suggests novel control points of antifungal defence and resistance

The general assumption is that biotic stress such as herbivory or infection by necrotrophic microbes triggers rapid JA-Ile hormone accumulation, and, in turn, a simple transcriptional model predicts that the defence response amplitude and subsequent resistance track hormone levels. This has been verified in many studies, but the present data identify new situations where more complexity emerges, and distorts the linear relationship between JA-Ile levels and defence/resistance strength.

Two distinct scenarios may underlie the characteristics of CYP94 KO and CYP94 OE plants: cyp94 KO plants accumulate more JA-Ile due to reduced catabolism, yet they do not exhibit a clear gain in fungal resistance. Possibly, JA-Ile levels are close to saturation in terms of defence signalling, even in the WT, and a moderate hormone increase in KOs only generates a negligible increase in PDF1.2 and PR4 expression, insufficient to improve resistance. The transiently increased ERF1 signal in KOs (2 dpi) confirmed a higher JA-Ile input, which was not translated into more defence. The inability of CYP94-deficient plants to durably deploy higher defence and resistance phenotypes may find an explanation in the prolonged overinduction of specific JAZ genes such as JAZ1 and JAZ5. Although this has to be established at the protein and target promoter levels, it is likely that some JAZ genes react to abnormal JA-Ile increases in a negative-feedback loop to prevent excessive downstream target responses. As a consequence, increasing crop tolerance to necrotrophs by inhibiting JA-Ile catabolism will require more refined strategies.

In marked contrast, both OE plant lines showed clearly impaired antifungal resistance. In the case of B₃-OE, this phenotype could be explained by reduced JA-Ile levels due to exacerbated catabolic inactivation that attenuates defence. The situation with C₁-OE was more unexpected, as this line showed essentially normal JA-Ile accumulation, and depressed JAZ8 and JAZ9 expression, yet it bore the most severe B. cinerea symptoms and multiplication, along with largely impaired defence responses. The observation that specific JAZ genes are antagonistically affected when either blocking or enhancing CYP94 expression suggests differential JAZ sensitivity to alterations in the hormone oxidation status. This may reveal a repression rheostat to allow flexible control in the signalling output by adapting JAZ repressor strength to actual JA-Ile turnover. Because OE plants are characterized by their high content of oxidized hormone, a possibility is that JA-Ile levels are not the sole hormonal factor determining defence output, and that the simultaneous presence of abundant 12-OH-JA-Ile and/or 12-COOH-JA-Ile catabolites negatively affects signalling through an unknown mechanism.

Another aspect revealed here concerns the interaction of JA-Ile catabolism with SA signalling. In OE plants, the JA signalling pathway is largely disabled, and consequently, this relieves the repression on the SA pathway, as attested by higher SA content and PR1 marker expression. Predictively, this shift towards more SA defence in OE plants should lead to enhanced resistance towards biotrophic pathogens. This prediction is supported by the work of Hwang and Hwang (2010), who showed that cyp94b₃ mutants are more susceptible to the hemi-biotrophic bacterial pathogen P. syringae DC3000. In the same study, Arabidopsis plants ectopically expressing a pepper CYP94B₃ homologue were more resistant to the obligate biotrophic oomycete Hyaloperonospora arabidopsidis (Hwang and Hwang, 2010). These results show that perturbing JA-Ile turnover has distinct consequences on pathogens with different lifestyles.

CYP94-mediated JA-Ile oxidation gradually impairs co-receptor assembly and signalling properties

Finally, we addressed the direct signalling capacities of JA-Ile and its two CYP94-generated derivatives by external treatment of seedlings, an approach that had not been explored so far. Hydroxylation was sufficient to remove most but not all gene-inducing activity, while the carboxy derivative lost any detectable activity. We cannot rule out that oxidation status could influence the uptake of compounds by seedlings. The most likely hypothesis is that oxidation alters the capacity of conjugates to act as ligands promoting co-receptor assembly and subsequent signalling, and this was supported by the in vitro pull-down assay with JAZ9, extending similar conclusions reported for other JAZ proteins (Koo et al., 2014). The highly polar environment at the C₁₂ carbon in 12-COOH-JA-Ile may prevent interaction with the hydrophobic pocket of the receptor that accommodates JA-Ile (Sheard et al., 2010), but this needs experimental validation. We finally considered the possibility that an excess of oxidized derivatives may inhibit the signalling action of JA-Ile, providing a basis for understanding the impaired defence output in Botrytis-infected B₃-OE and C₁-OE plants, which overaccumulate these compounds. In preliminary experiments, concomitant application of JA-Ile along with an excess of oxidized derivatives did not strongly reduce JA-Ile gene-inducing activity. Therefore, understanding the molecular basis underlying impairment of JA-Ile-dependent defences in CYP94-OE plants will require further investigation.

Supplementary data

Supplementary data are available at JXB online.

Supplementary Fig. S1. IAR3 and ILL6 amidohydrolase expression profiles in WT plants in response to B. cinerea.
Supplementary Fig. S2. Expression characteristics of B3-OE and bhlh3-1 mutant lines.

Supplementary Fig. S3. Expression profiles of the 12 Arabidopsis JAZ genes in WT plants in response to B. cinerea infection.

Supplementary Table S1. Primers used in this study.

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