Catabolism and deactivation of the lipid-derived hormone jasmonoyl-isoleucine

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The oxylipin hormone jasmonate controls myriad processes involved in plant growth, development, and immune function. The discovery of jasmonoyl-L-isoleucine (JA-Ile) as the major bioactive form of the hormone highlights the need to understand biochemical and cell biological processes underlying JA-Ile homeostasis. Among the major metabolic control points governing the accumulation of JA-Ile in plant tissues are the availability of jasmonic acid, the immediate precursor of JA-Ile, and oxidative enzymes involved in catabolism and deactivation of the hormone. Recent studies indicate that JA-Ile turnover is mediated by a ω-oxidation pathway involving members of the CYP94 family of cytochromes P450. This discovery opens new opportunities to genetically manipulate JA-Ile levels for enhanced resistance to environmental stress, and further highlights ω-oxidation as a conserved pathway for catabolism of lipid-derived signals in plants and animals. Functional characterization of the full complement of CYP94 P450s promises to reveal new pathways for jasmonate metabolism and provide insight into the evolution of oxylipin signaling in land plants.

Keywords: oxylipin metabolism, lipid signaling, cytochrome P450, *Arabidopsis*, plant hormone, plant defense, jasmonate, omega oxidation

INTRODUCTION

Plants use a wide variety of lipid-based signals to control fundamental aspects of growth, development, and responses to environmental stress. Among the most intensively studied of these signals are members of the jasmonate family of oxylipins, collectively referred to as JAs. JAs are biochemically defined as cyclopente(a)none compounds derived from lipoxygenase-dependent oxidation of polyunsaturated fatty acids. Jasmonic acid (JA) and its derivatives are well known for their role in orchestrating immune responses to a broad spectrum of arthropod herbivores and microbial pathogens (Glazebrook, 2005; Wasternack, 2007; Browse and Howe, 2008; Howe and Jander, 2008; Wu and Baldwin, 2010). JAs also serve important roles in plant growth and development, including sexual reproduction, growth control, and secondary metabolism (McConn and Browse, 1996; Li et al., 2004; Yan et al., 2007; Pauwels et al., 2008; Zhang and Turner, 2008). Increasing evidence indicates that the JA pathway participates in extensive crosstalk with other hormones that mediate developmental plasticity (Dombrecht et al., 2007; Moreno et al., 2009; Pieterse et al., 2009; Hou et al., 2010; Robson et al., 2010; Ballare, 2011; Kazan and Manners, 2011; Zhu et al., 2011). Collectively, these studies support the view that JAs control resource allocation between growth- and defense-related processes, thus allowing plants to rapidly adapt to changing environmental conditions. A greater understanding of JA homeostasis is therefore relevant to many areas of plant physiology (Howe, 2010; Kazan and Manners, 2012).

Significant recent progress has been made in understanding the molecular mechanism by which JAs control large-scale changes in gene expression in response to stress. In unstressed cells containing low levels of the hormone, transcription factors (e.g., MYC2) that promote expression of JA-responsive genes are repressed by members of the JASMONATE ZIM-domain (JAZ) protein family (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007). In response to stress-related cues that trigger JA accumulation, bioactive forms of the hormone stimulate JAZ binding to CORONATINE INSENSITIVE1 (COI1), which is the F-box protein component of the E3 ubiquitin ligase SCF^{COI1} (Xie et al., 1998; Thines et al., 2007; Katsir et al., 2008b; Melotto et al., 2008; Fonseca et al., 2009; Yan et al., 2009). Proteolytic degradation of JAZ via the ubiquitin/26S proteasome pathway releases JAZ-bound transcription factors from repression, thereby allowing expression of JA-responsive genes.

A major challenge in the field of plant lipid signaling is to identify biologically active oxylipins and their cognate receptors. Significant insight into this question came from the discovery that the interaction of COI1 with its JAZ substrates is promoted in a highly specific manner by the isoleucine-conjugated form of JA (jasmonoyl-L-isoleucine, JA-Ile), but not by free JA or other non-conjugated JAs (Thines et al., 2007; Katsir et al., 2008b; Melotto et al., 2008; Fonseca et al., 2009; Yan et al., 2009). X-ray crystallography studies demonstrated that the JA and Ile moieties of JA-Ile serve critical roles in the assembly of COI1–JAZ receptor complexes, as does an inositol pentakisphosphate cofactor that interacts with COI1 and JAZ near the hormone-binding pocket (Sheard et al., 2010). The key role of JA-Ile in promoting JAZ degradation is consistent with genetic studies that established a role for the JA-conjugating enzyme JAR1 in JA-mediated physiological processes (Staswick et al., 1998; Staswick and Tiryaki, 2004; Kang et al., 2006; Koo et al., 2009).

For the purposes of this review, we define bioactive JAs as derivatives that promote COI1–JAZ interactions to affect transcriptional responses. Non-bioactive JAs are either precursors or deactivated (or less active) forms of bioactive JAs. Below, we summarize the current understanding of metabolic pathways involved in catabolism and deactivation of JA-Ile, with special attention given to the recently discovered cytochrome P450-mediated ω-oxidation pathway.

OVERVIEW OF JA-ILE METABOLISM

The initial stage of JA biosynthesis occurs in the plastid and involves the conversion of α-linolenic acid (18:3) to 12-oxophytodienoic acid (OPDA; **Figure 1**). Following transport to the peroxisome, OPDA is reduced to its cyclopentanone derivative (OPC-8:0) and subjected to three cycles of β-oxidation to yield the 3R,7S stereoisomer of JA [also known as (+)-7-iso-JA]. JA is transported to the cytosol where it is conjugated specifically to Ile by the enzyme JAR1 (Staswick and Tiryaki, 2004; Suza and Staswick, 2008). JA-Ile synthesized in the cytosol presumably diffuses into the nucleus where it binds COI1–JAZ receptor complexes to activate gene expression (**Figure 1**).

The biosynthesis of JA-Ile is tightly controlled by developmental and environmental cues (Creelman and Mullet, 1997; Wasternack, 2007; Koo and Howe, 2009). Consistent with their role in regulating induced defenses in vegetative tissues, JA and JA-Ile typically accumulate in response to various biotic and abiotic stresses. Mechanical wounding of *Arabidopsis* leaves, for example, effectively triggers *de novo* accumulation of JA/JA-Ile within minutes of tissue damage (Chung et al., 2008; Glauser et al., 2008; Suza and Staswick, 2008; Koo and Howe, 2009; Koo et al., 2009; Suza et al., 2010). The rapidity of this response indicates that all JA-Ile

biosynthetic enzymes, including JAR1, are present in unstressed cells prior to stimulation. This view is consistent with studies showing that the major rate-limiting step in JA/JA-Ile synthesis is lipase-mediated release of fatty acyl substrates from plastid glycerolipids (Ishiguro et al., 2001; Stenzel et al., 2003; Wasternack, 2007; Kallenbach et al., 2010; Bonaventure et al., 2011). The mechanism by which extracellular signals activate plastidic lipases to trigger JA-Ile formation is a major unanswered question. Genes encoding many of the enzymes involved in JA-Ile biosynthesis are coordinately up-regulated in response to environmental signals that activate production of the hormone (Reymond et al., 2000; Sasaki et al., 2001; Sasaki-Sekimoto et al., 2005; Koo et al., 2006; Ralph et al., 2006; Pauwels et al., 2008). This transcriptional response presumably constitutes a positive feedback mechanism to amplify the cell's capacity for JA metabolism. At a practical level, this co-expression phenomenon has proven useful for the identification of novel components in the JA metabolic and signaling pathways (Koo et al., 2006, 2011; Thines et al., 2007; Heitz et al., 2012).

In addition to the availability of plastid-derived fatty acyl substrates, there is evidence that the rate of JA-Ile biosynthesis is influenced by metabolic pathways that compete with JAR1 for cytosolic pools of JA. This idea is consistent with the fact that stress-induced levels of JA-Ile are typically well below (∼10%) that of JA (Kang et al., 2006; Suza and Staswick, 2008; Koo et al., 2009). Among the metabolic pathways that potentially divert JA from JA-Ile biosynthesis are those involved in formation of JA-glucose esters (Swiatek et al., 2004), 12-hydroxy-JA (12-OH-JA) and its sulfated and glycosylated derivatives (Gidda et al., 2003; Miersch et al., 2008), volatile methyl-JA (MeJA), and JA-amino acid conjugates other than JA-Ile (Wang et al., 2007; **Figure 1**). Studies involving
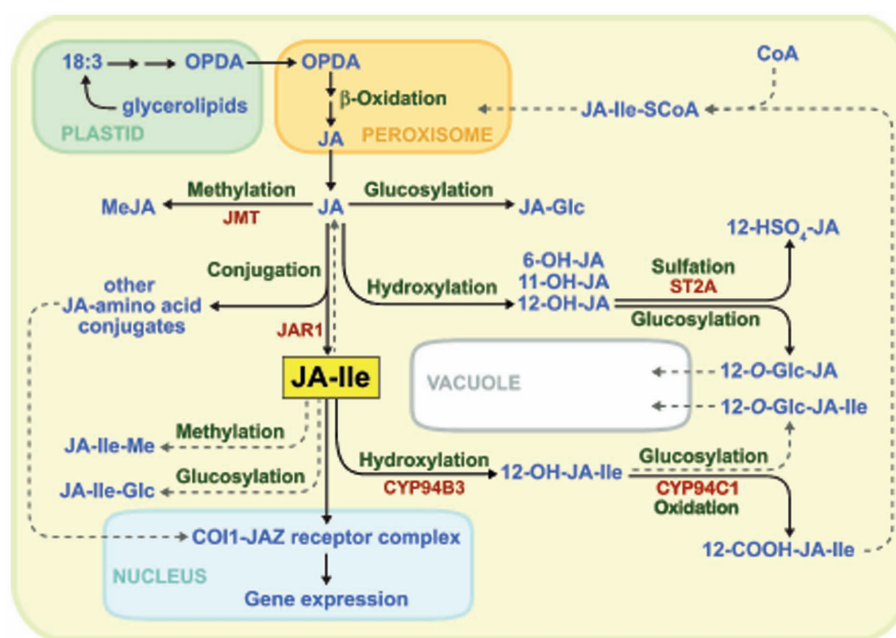

FIGURE 1 | Major pathways for the biosynthesis and catabolism of JA-Ile. Confirmed biochemical pathways and products are indicated by solid lines, whereas dashed lines denote hypothetical pathways for which there is currently little or no evidence. See text for details.

ectopic expression of an *Arabidopsis* JA carboxyl methyltransferase (JMT) in *Nicotiana attenuata* provided genetic evidence that increased flux of JA into MeJA has predicted negative effects on JA-Ile formation and JA-Ile-mediated physiological process (Stitz et al., 2011). These findings, together with the inability of JA and MeJA to promote COI1–JAZ binding (Thines et al., 2007), provide convincing evidence that JA and MeJA are non-bioactive precursors of JA-Ile. The ability of exogenous JA and MeJA to potently activate hormonal responses can be attributed to their *in vivo* conversion to JA-Ile and subsequent action through the COI1–JAZ receptor system (Tamogami et al., 2008; Wu et al., 2008).
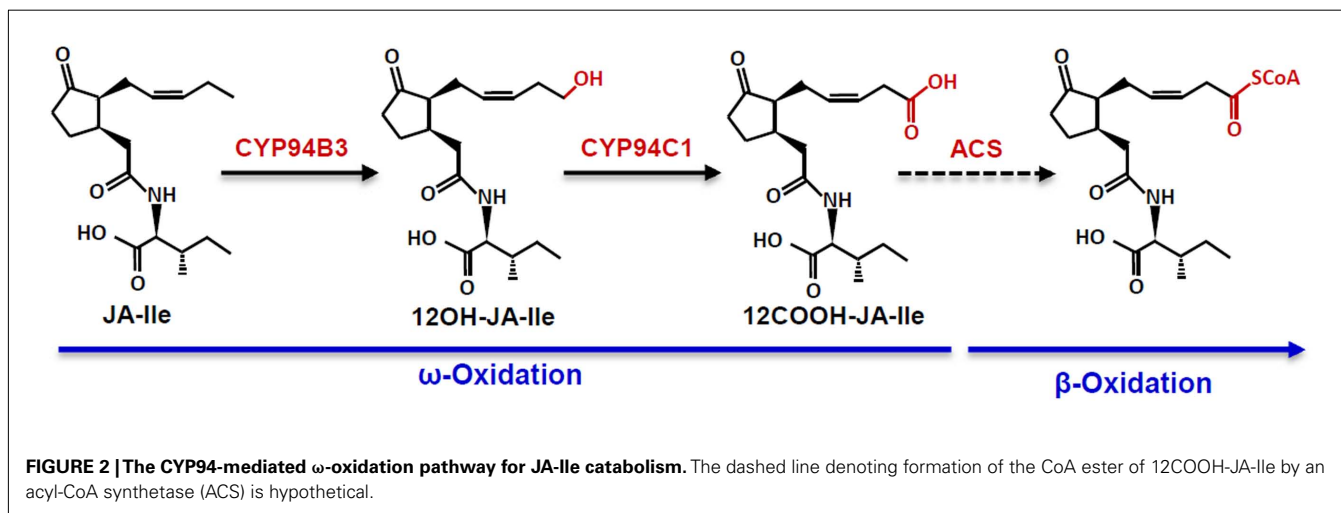
The transient accumulation of JA-Ile in injured leaves is tightly correlated with the expression of primary JA-response genes (Chung et al., 2008; Koo et al., 2009). This observation implies the existence of pathways for catabolism and deactivation of the hormone. As is the case for most stress hormones in plants and animals, such pathways serve important roles in attenuating physiological outputs during the stress response or for switching off the response when stress levels have subsided. The ω-oxidation pathway, in which JA-Ile is converted to 12-hydroxy-JA-Ile (12-OH-JA-Ile) and then further oxidized to dicarboxy-JA-Ile (12-COOH-JA-Ile), is now recognized as a major route for catabolism of the hormone (**Figures 1** and **2**; Guranowski et al., 2007; Glauser et al., 2008; Miersch et al., 2008; Kitaoka et al., 2011; Koo et al., 2011; VanDoorn et al., 2011; Heitz et al., 2012). Genetic evidence (see below), together with the finding that 12-OH-JA-Ile is less effective than JA-Ile in promoting COI1–JAZ interaction *in vitro* (Koo et al., 2011), firmly establishes a functional role for ω-oxidation in attenuating JA-Ile-mediated signaling. Pathways involved in further metabolism of oxidized JA-Ile derivatives are likely to exist but have not yet been reported. One possibility is formation of *O*-linked glucosyl derivatives of 12-OH-JA-Ile, which may be rendered ineffective as a ligand for the COI1–JAZ receptor, or perhaps sequestered in the vacuole. It is also possible that the newly formed carboxyl group of 12-COOH-JA-Ile is esterified to CoA and subsequently subject to β-oxidation in the peroxisome (**Figures 1** and **2**). This hypothesis is supported by the existence of β-oxidization enzymes that metabolize unusual fatty acid substrates, including those containing unsaturated bonds such as that

found in the pentenyl side chain of JA-Ile (Goepfert and Poirier, 2007).

The turnover of endogenous JA-Ile in mutants that are impaired in JA-Ile 12-hydroxylation suggests that additional pathways may participate in JA-Ile metabolism (**Figure 1**; Koo et al., 2011). Among the possible alternative metabolic routes of hormone deactivation are formation of JA-Ile glucose esters and, by analogy to the role of amidohydrolases in auxin homeostasis (LeClere et al., 2002; Woodward and Bartel, 2005), hydrolysis of JA-Ile to free JA and Ile. It has also been suggested that esterification of JA-Ile to JA-Ile-Me, as well as epimerization of (3*R*,7*S*)-JA-Ile to the less active (3*R*,7*R*) isomer, may be endogenous mechanisms to reduce the activity of the hormone; both of these products are largely inactive in promoting COI1–JAZ interaction *in vitro* (Fonseca et al., 2009). Recent *in vivo* studies, however, indicate that epimerization of (3*R*,7*S*)-JA-Ile is unlikely to play a significant role in deactivation of JA-Ile (Suza et al., 2010).

ROLE OF CYP94 P450s IN CATABOLISM OF BIOACTIVE JAs

In contrast to detailed knowledge of nearly all genes encoding the core set of JA-Ile biosynthetic enzymes (Wasternack, 2007; Schaller and Stintzi, 2009), understanding the genetic basis of JA-Ile catabolism is still a largely unexplored area of plant hormone research. The pervasive role of cytochrome P450 hydroxylases in the deactivation of small-molecule hormones suggest that P450s also participate in catabolism of JA-Ile. Based on this assumption, Koo et al. (2011) focused attention on members of the CYP86 clan of P450s in *Arabidopsis* that play a prominent role in fatty acid hydroxylation, including ω-hydroxylation. Gene co-expression analysis was used to narrow the list of candidates to three *CYP94* genes (*CYP94B1*, *CYP94B3*, and *CYP94C1*) whose expression is induced in response to wounding and JA treatment. Various members of this group were previously shown to be regulated by the JA pathway, and to encode enzymes that hydroxylate straight-chain fatty acids *in vitro* (Duan and Schuler, 2005; Benveniste et al., 2006; Kandel et al., 2007; Ehlting et al., 2008). Analysis of T-DNA-tagged mutants showed that disruption of *CYP94B3*, but not *CYP94B1* or *CYP94C1*, results in a metabolic phenotype indicative of a defect in JA-Ile 12-hydroxylase activity; in

FIGURE 2 | The CYP94-mediated ω-oxidation pathway for JA-Ile catabolism. The dashed line denoting formation of the CoA ester of 12COOH-JA-Ile by an acyl-CoA synthetase (ACS) is hypothetical.

response to wounding, *cyp94b3* mutants hyperaccumulate JA-Ile and are also deficient in 12-OH-JA-Ile. The biochemical function of CYP94B3 was confirmed by experiments showing that the recombinant enzyme has JA-Ile 12-hydroxylase activity (Koo et al., 2011). Independent studies confirmed the identity of CYP94B3 as a JA-Ile 12-hydroxylase and further demonstrated that CYP94C1 plays a major role in conversion of 12-OH-JA-Ile to 12-COOH-JA-Ile (Kitaoka et al., 2011; Heitz et al., 2012). These collective studies establish a central role for CYP94s in the ω-oxidation pathway for turnover and deactivation of JA-Ile (**Figure 2**). Interestingly, CYP P450-mediated ω-oxidation (and subsequent β-oxidation) pathways also mediate catabolism and deactivation of eicosanoid signals in animal cells (Hardwick, 2008; Buczynski et al., 2009). The discovery of the ω-oxidation pathway for JA-Ile catabolism therefore highlights a conserved feature of lipid-based signaling in plants and animals.

The phenotype of mutants affected in CYP94B3 expression demonstrates the importance of JA-Ile catabolism in plant growth, development, and defense. CYP94B3-overexpressing plants, which are severely deficient in wound-induced JA-Ile accumulation, display hallmark signs of JA-Ile deficiency, including defects in anther and pollen development, resistance to JA-mediated root growth inhibition, and increased susceptibility to insect herbivores (Koo et al., 2011). In contrast, hyperaccumulation of JA-Ile in *cyp94b3* null mutants results in increased sensitivity to the hormone. Among the phenotypes reported for *cyp94b3* single and *cyp94b3cyp94c1* double mutants are enhanced expression of JA-response genes and increased sensitivity to JA-mediated root growth inhibition (Kitaoka et al., 2011; Koo et al., 2011; Heitz et al., 2012). It was also reported that *cyp94b3* mutants are more susceptible to a virulent strain of *Pseudomonas syringae* (Hwang and Hwang, 2010), which may reflect the fact that increased signaling through the JA-Ile receptor compromises host resistance to this pathogen by suppressing the salicylate-mediated branch of immunity (Glazebrook, 2005; Katsir et al., 2008b). These collective results demonstrate that CYP94B3 and CYP94C1 function to reduce the active pool of JA-Ile, and that this route of hormone catabolism affects physiological processes in roots, leaves, and reproductive tissues. This conclusion is consistent with the emerging view that JA-Ile is the active signal for many, if not most, jasmonate responses.

MINING THE CYP94ome

Cytochrome P450s constitute the most metabolically diverse group of enzymes in the plant kingdom (Nelson and Werck-Reichhart, 2011). The recent characterization of CYP94B3 and CYP94C1 (Kitaoka et al., 2011; Koo et al., 2011; Heitz et al., 2012) highlight the widespread role of P450s in plant hormone metabolism (Mizutani and Ohta, 2010) and shed light on the physiological function of the broader CYP94 family. *Arabidopsis* contains six CYP94 members belonging to three subfamilies: CYP94B1, CYP94B2, CYP94B3, CYP94C1, CYP94D1, and CYP94D2 (**Figure 3**). That various CYP94 subfamilies have undergone significant expansion and contraction in particular angiosperm lineages suggests the existence of rapidly evolving metabolic pathways involving fatty acid-derived compounds (Guttikonda et al., 2010; Nelson and Werck-Reichhart, 2011).
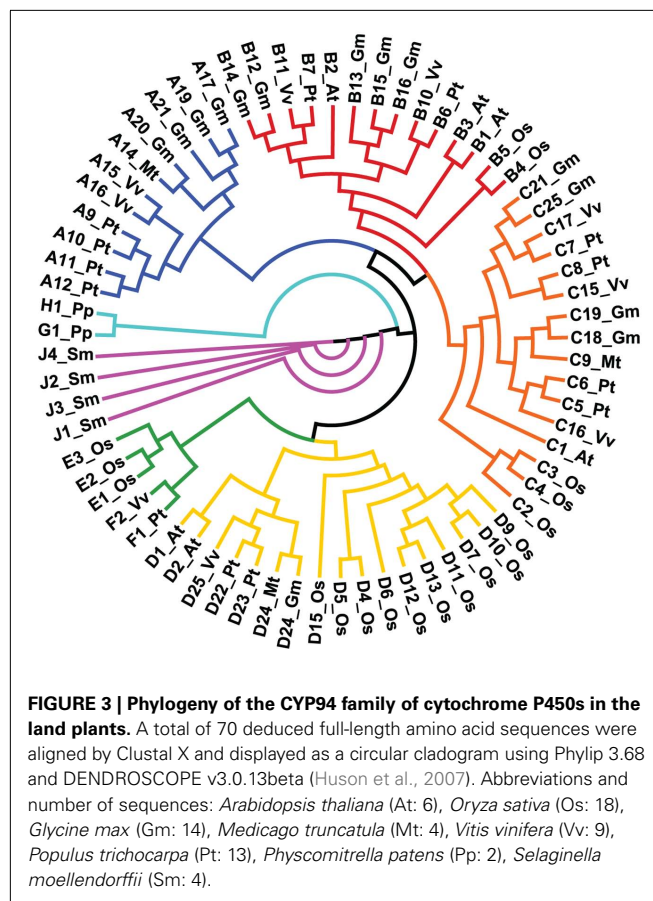

FIGURE 3 | Phylogeny of the CYP94 family of cytochrome P450s in the land plants. A total of 70 deduced full-length amino acid sequences were aligned by Clustal X and displayed as a circular cladogram using Phylip 3.68 and DENDROSCOPE v3.0.13beta (Huson et al., 2007). Abbreviations and number of sequences: *Arabidopsis thaliana* (At: 6), *Oryza sativa* (Os: 18), *Glycine max* (Gm: 14), *Medicago truncatula* (Mt: 4), *Vitis vinifera* (Vv: 9), *Populus trichocarpa* (Pt: 13), *Physcomitrella patens* (Pp: 2), *Selaginella moellendorffii* (Sm: 4).

P450 phylogenies also indicate that the CYP94 family is conserved in non-vascular plants (e.g., *Physcomitrella patens*) but is not present in photosynthetic aquatic organisms. This distribution of CYP94s coincides with the occurrence in these lineages of genes encoding various components of the JA biosynthesis and signaling pathways (Chico et al., 2008; Katsir et al., 2008a; Browse, 2009). Interestingly, *P. patens* contains plastidic enzymes for the synthesis of OPDA but appears to lack the ability to produce JA and JA-Ile (Stumpe et al., 2010). Functional characterization of CYP94 P450s in *P. patens* may thus provide insight into the evolutionary origins of oxylipin signaling and metabolism.

The role of CYP94B3 and CYP94C1 in JA-Ile ω-oxidation suggests that other CYP94s serve similar roles in oxylipin metabolism. For example, the inability of CYP94B3 and C1 to hydroxylate JA *in vitro* (Kitaoka et al., 2011; Heitz et al., 2012) raises the possibility that formation of 12-OH-JA is catalyzed by another CYP94 member. The presence of residual amounts of 12-OH-JA-Ile in *cyp94b3* and *cyp94b3cyp94c1* mutants (Kitaoka et al., 2011; Koo et al., 2011; Heitz et al., 2012) further suggests that additional enzymes participate in 12-hydroxylation of JA-Ile. Given the broad specificity of many CYP94s for fatty acyl substrates *in vitro* (Benveniste et al., 2006; Kandel et al., 2007; Pinot and Beisson, 2011; Heitz et al., 2012), functional characterization of additional family members will clearly benefit from analysis of metabolic phenotypes of *cyp94* mutants.

SUMMARY AND FUTURE PERSPECTIVES

It is becoming increasing clear that the JA signaling pathway is embedded in a complex phytohormone network that controls myriad aspects of plant growth and development, as well as the way in which plants adapt to their environment. Current models of JA signal transduction support the view that the intracellular level of JA-Ile plays a major role in controlling the strength of JA responses. Recent studies with mutants that are altered in CYP94 expression demonstrate the importance of JA-Ile catabolism in attenuating this hormone response pathway (Kitaoka et al., 2011; Koo et al., 2011; Heitz et al., 2012). Negative feedback control of JA responses also depends on other mechanisms, including JA-induced expression of stabilized JAZ isoforms that fail to efficiently bind COI1 in the presence of JA-Ile (Chung and Howe, 2009; Chung et al., 2010; Shyu et al., 2012). Further elucidation of these negative feedback pathways may facilitate biotechnological efforts aimed at exploiting the JA pathway for enhanced resistance of crop plants to insects and pathogens, and for producing economically important plant compounds (e.g., paclitaxel) whose expression is controlled by the JA pathway. Several additional gaps in our understanding of how JA-Ile levels are regulated also remain to be addressed. For example, there is a pressing need to understand the early signaling events that trigger JA-Ile synthesis, and to fully elucidate metabolic pathways that govern deactivation of the hormone. Progress toward the latter goal will undoubtedly be facilitated by further characterization of the "CYP94ome." Given the high degree of sequence similarity between CYP94s and CYP86 P450s that catalyze the formation of cutin and wax polymers of the epidermal cuticle (Pinot and Beisson, 2011), this line of research may also provide insight into the evolutionary relationship between oxylipins that serve signaling *versus* structural roles in plant defense.

Another promising area of future research will be to discern the spatial and temporal distribution of JAs in different tissues and cell types. Existing approaches to study the tissue-specific accumulation (Hause et al., 2000; Glauser et al., 2008; Stitz et al., 2011) and non-cell autonomous action (Li et al., 2002; Koo et al., 2009) of JAs would benefit from new technologies to quantify hormone levels with cellular and sub-cellular resolution (Bermejo et al., 2011; Mielke et al., 2011). Such efforts are expected to lead to a better understanding of how bioactive and non-bioactive JAs are compartmentalized within cells and transported between cells. Given the plethora of JA derivatives produced in plants, it will also be important to identify additional bioactive JAs that exert physiological effects, either through the COI1–JAZ receptor system or via other mechanisms. For example, the identity of 12-OH-JAs as factors for leaf closing (Nakamura et al., 2011) and tuber induction (Yoshihara et al., 1989) provides tantalizing evidence that JAs evolved as lipid-derived signals for regulating specialized aspects of plant physiology.

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