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Biosynthesis and Metabolism of Jasmonates

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Abstract

Jasmonates are derived from oxygenated fatty acids via the octadecanoid pathway and characterized by a pentacyclic ring structure. They have regulatory function as signaling molecules in plant development and adaptation to environmental stress. Until recently, it was the cyclopentanone jasmonic acid (JA) that attracted most attention as a plant growth regulator. It becomes increasingly clear, however, that biological activity is not limited to JA but extends to, and may even differ between its many metabolites and conjugates as well as its cyclopentenone precursors. The enzymes of jasmonate biosynthesis and metabolism may thus have a regulatory function in controlling the activity and relative levels of different signaling molecules. Such a function is supported by both the characterization of loss of function mutants in *Arabidopsis*, and the biochemical characterization of the enzymes themselves.

Key words: Jasmonates; Octadecanoid pathway; Oxylipins; Plant defense; Plant development; Plant hormones; Signaling

Introduction

Since the initial discovery of methyl jasmonate (MeJA) as a secondary metabolite in essential oils of jasmin in 1962 (Demole and others 1962), jasmonates have come a long way and are now recognized as a genuine class of plant hormones. In the early 1980s, their widespread occurrence throughout the plant kingdom (Meyer and others 1984) and their growth-inhibitory (Dathe and others 1981) and senescence-promoting activities (Ueda and Kato 1980) were established. The term jasmonates, at that time, referred to jasmonic acid (JA) and its methyl ester, and biological activity was mostly attributed to the free acid (Farmer and Ryan 1992; Sembdner and Parthier 1993). It has been shown in recent years and will be further emphasized in the present article that bioactivity is not limited to JA. Many related compounds including JA precursors and conjugates, which are all characterized by a pentacyclic ring structure and their biosynthetic origin from oxygenated polyunsaturated fatty acids (oxylipins), have been shown to have biological activity *per se* (Kramell and others 1997; Stintzi and others 2001), and are here collectively referred to as jasmonates.

Also already in the early 1980s, the pathway for JA biosynthesis was elucidated by Vick and Zimmerman. JA was shown to be synthesized from linolenic acid, which is first oxygenated by lipoxygenase (LOX), to yield 13(*S*)-hydroperoxy linolenic acid (13-HPOT) (Vick and Zimmerman 1984). Zimmer-

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man and Feng observed that this fatty acid hydroperoxide can be cyclized to 12-oxophytodienoic acid (OPDA) (Zimmerman and Feng 1978) and the apparent hydroperoxide cyclase activity was found to be present in many plant species (Vick and Zimmerman 1979). We know now that the cyclization is achieved by the consecutive action of two enzymes, namely, allene oxide synthase (AOS) and allene oxide cyclase (AOC; Figure 1). Vick and Zimmerman continued to show that OPDA is further metabolized to JA. This includes the reduction of the cyclopentenone ring of OPDA to yield the respective cyclopentanone (OPC 8:0) followed by three cycles of β-oxidation resulting in the shortening of the octanoic acid side chain and the formation of JA (Vick and Zimmerman 1984) (Figure 1). Research in recent years generally confirmed the Vick and Zimmerman pathway of JA biosynthesis (the octadecanoid pathway) and considerable progress was made with respect to the biochemistry of the enzymes involved, as well as the molecular organization and regulation of the pathway. Furthermore, fatty acid-derived hydroperoxides were found to be precursors not only of jasmonates but of many other bioactive oxylipins. In addition to the octadecanoid pathway, seven different routes for oxylipin biosynthesis were shown to exist (Figure 2) (reviewed by Blee 1998; Feussner and others 2001; Schaller 2001; Howe and Schilmiller 2002). In the present review we will focus on just one of these routes, the one for jasmonate biosynthesis, with emphasis on its regulation, the enzymes involved, and the activity of the metabolites.

The early elucidation of the jasmonate biosynthetic pathway and the demonstration of their growth-inhibiting and senescence-promoting activity were followed by the discovery that jasmonates are involved in plant defense reactions, which greatly stimulated the interest in these compounds as plant signaling molecules. A role in plant defense was first shown by Farmer and Ryan who demonstrated the induction of proteinase inhibitors by MeJA and JA as part of the defense response against herbivorous insects (Farmer and Ryan 1990; Farmer and others 1991). Jasmonates were then shown to be active inducers of antimicrobial phytoalexins by Zenk and coworkers (Gundlach and others 1992) and subsequent work clearly established their defense gene-inducing activity for enhanced resistance against insect predators and pathogens (reviewed by Reymond and Farmer 1998; Weiler and others 1998; Blee 2002).

Such a role was unequivocally confirmed by the analysis of mutants compromised in either the synthesis or the perception of jasmonate signals. The

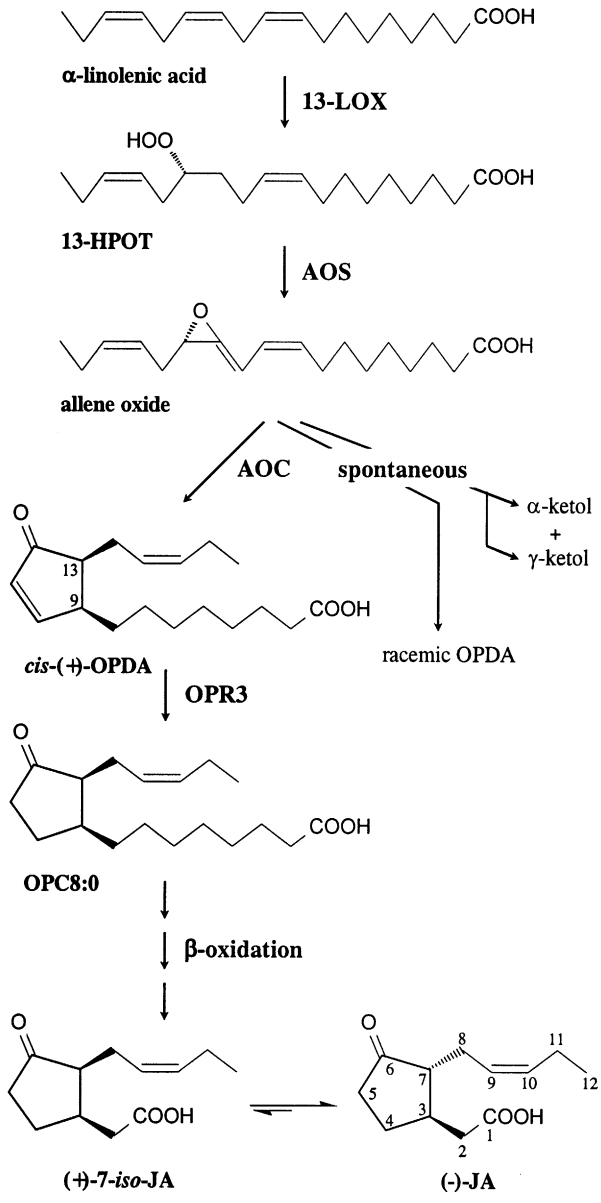

Figure 1. The Vick and Zimmerman pathway for JA biosynthesis. Pathway intermediates are abbreviated as 13-HPOT for (9Z11*E*15Z13S)-13-hydroperoxy-9,11,15-octadecatrienoic acid (that is, 13(S)-hydroperoxy linolenic acid), allene oxide for (12,13(S)-epoxy-9(Z),11,15(Z)-octadecatrienoic acid, *cis*-(+)-OPDA for *cis*-(+)-12-oxophytodienoic acid, and OPC8:0 for 3-oxo-2(2'(Z)-pentenyl)-cyclopentane-1-octanoic acid. The enzymes are indicated as 13-LOX for 13-lipoxygenase, AOS for allene oxide synthase, AOC for allene oxide cyclase, and OPR3 for 12-oxophytodienoate reductase 3. (Figure adapted from Wasternack and Hause 2002)

regulation of insect and pathogen defense gene expression is affected in mutants that fail to respond to JA like the *coi1*, the *jar 1* and the *jin1* mutants in *Arabidopsis* (Berger and others 1996; Xie and others

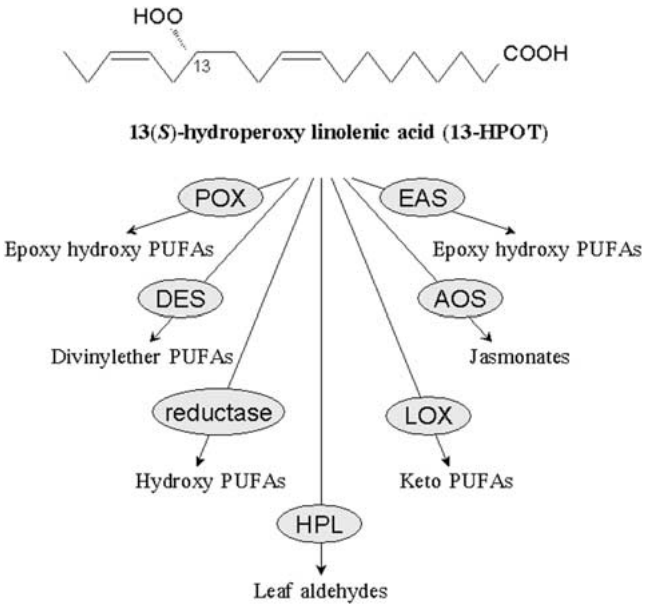

Figure 2. The metabolic fate of fatty acid hydroperoxides. Fatty acid hydroperoxides (only the 13(*S*)-hydroperoxy derivative of linolenic acid is shown) are channeled into seven distinct pathways for oxylipin biosynthesis. The committed steps are catalyzed by AOS (allene oxide synthase) for jasmonate biosynthesis, DES (divinylether synthase) for the formation of divinyl ethers, EAS (epoxyalcohol synthase) and POX (peroxygenase) resulting in epoxy hydroxy polyunsaturated fatty acids (PUFAs), HPL (hydroperoxide lyase) for the production of leaf aldehydes (green leaf volatiles), LOX (lipoxygenase) for the formation of keto PUFAs, and hydroperoxide reductase in the formation of hydroxy PUFAs. (Figure adapted from Feussner and Wasternack 2002).

1998; Staswick and others 2002; Lorenzo and others 2004) or the *jai1* mutant in tomato (Li and others 2004). Likewise, mutants that are impaired in the production of trienoic fatty acids as precursors for JA synthesis (the *fad3fad7fad8* and the *spr2/LeFad7* mutants in *Arabidopsis* and tomato, respectively) are compromised in defense reactions (McConn and others 1997; Li and others 2003), as are mutants affected in the octadecanoid pathway itself (Howe and others 1996; Stintzi and others 2001; Park and others 2002). Interestingly, jasmonate biosynthesis and perception mutants in *Arabidopsis* are male sterile due to defects in anther and pollen maturation (Feys and others 1994; McConn and Browse 1996; Sanders and others 2000; Stintzi and Browse 2000; Ishiguro and others 2001; Park and others 2002; von Malek and others 2002) while sterility of *jasmonate insensitive 1* in tomato is caused by a defect in the maternal control of seed maturation (Li and others 2004).

These findings clearly show that jasmonates are indispensable not only for plant defense but also in plant development to complete the normal life cycle, and established them as genuine plant hormones. Other processes in which the hormonal function of jasmonates is undisputed include senescence, mechanotransduction in *Bryonia*, and tuberization in potato (Koda 1997; Weiler and others 1998). Notwithstanding the well-established role of jasmonates as signaling molecules in various different processes, the question of specificity of individual jasmonates remains open. Can they really be all alike in their activity? Several studies addressed the structure-activity relationship of jasmonates and, depending on the bioassay employed, striking differences were found with respect to the structural requirements for activity (Blechert and others 1997; Blechert and others 1999; Miersch and others 1999; Haider and others 2000). These findings suggested that different processes may actually be controlled by different jasmonates *in vivo*. Again, it was the analysis of mutants that provided unequivocal evidence for differences in the signaling properties of different members of the jasmonate family (Seo and others 2001; Stintzi and others 2001; Staswick and others 2002). In this article we will discuss the enzymes involved in the synthesis and metabolism of jasmonates and will focus on the ones controlling the relative levels and the activity of individual signaling molecules.

The Enzymes Involved in JA Biosynthsis

The substrate for the Vick and Zimmerman pathway and precursor for JA biosynthesis is linolenic acid (18:3) (LA). LA is present in cellular lipids where it originates from esterified oleic acid (18:1) which is successively converted to linoleic acid (18:2) and to 18:3 by membrane-bound fatty acid desaturases (Somerville and Browse 1991). Given that the early steps of JA biosynthesis are catalyzed by the chloroplast enzymes LOX, AOS, and AOC, the source of 18:3 is believed to be the chloroplast membranes. But what is actually the first reaction in jasmonate biosynthesis? The question is not as trivial as it may seem as there are three possible routes, all leading to JA, but differing with respect to the step at which esterified 18:3 (or its derivatives) isreleased from the membrane lipids (Figure 3). Either LA is liberated by an acylhydrolase or a phospholipase for subsequent oxygenation by a 13-LOX, or else, the order is reversed and a lipase cleaves 13-HPOT generated by a membrane-associated LOX acting on esterified 18:3. In the third scenario, lipid-bound 13- HPOT is further metabolized by AOS and AOC to yield

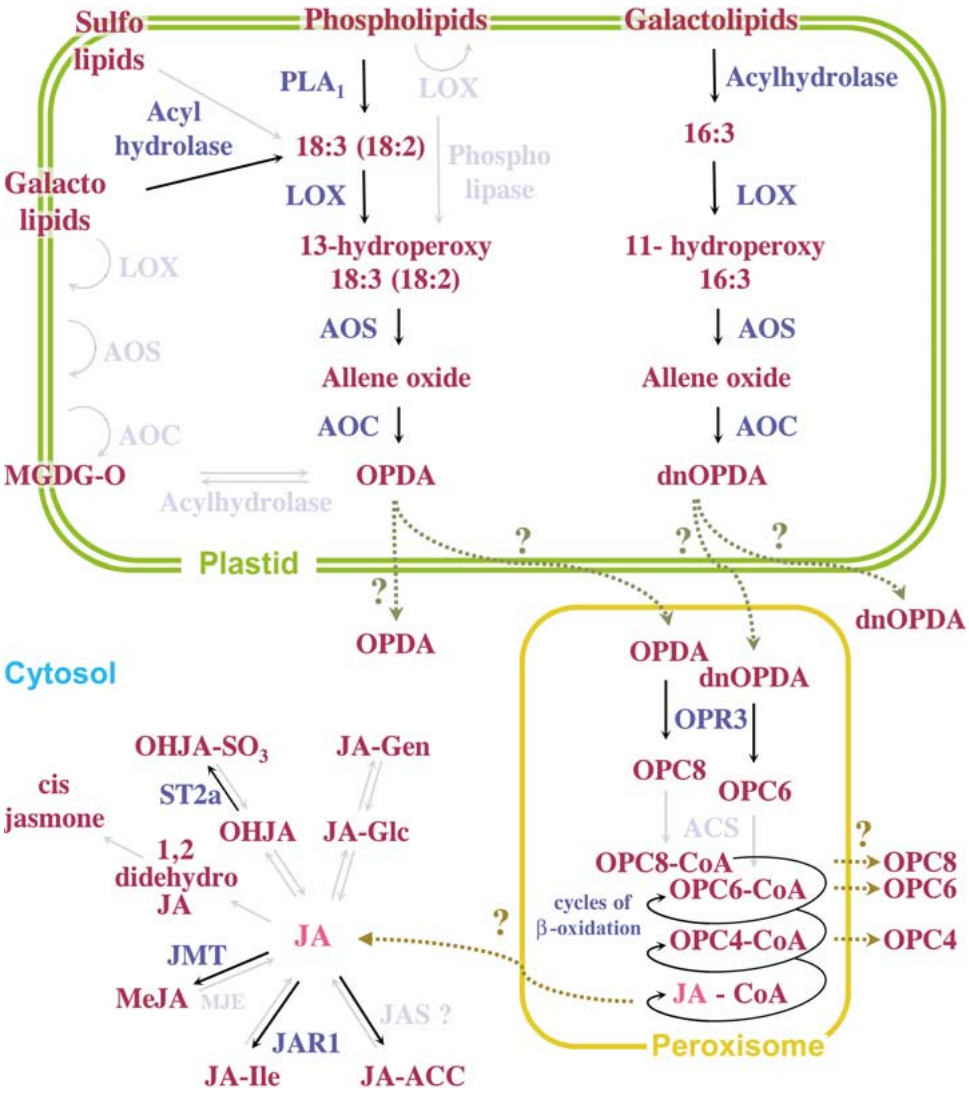

Figure 3. Overview of jasmonate biosynthesis and metabolism. JA biosynthesis and metabolism involve three compartments in the cell; that is, the chloroplast where OPDA and dnOPDA are synthesized, the peroxisome where (dn)OPDA is converted to JA, and the cytosol where further modifications of JA take place. Text and arrows in solid colors are supported by experimental evidence, hypothetical routes are depicted in lighter tones many of which involve membrane-localized esterified substrates. Transport of the various metabolites between compartments is indicated by dotted arrows and is hypothetical. For clarity of the figure, steps beyond 13-hydroperoxy linoleic acid were omitted but would lead to 9,10-dihydro-JA. Abbreviations are given in the text.

OPDA esterified to monogalactosyldiacylglycerol (MGDG-O), which may then be hydrolyzed by an acylhydrolase to result in free OPDA.

In the following, we will address the evidence for all three possibilities. Because most of the data support the lipolytic release of 18:3 as the initial step in JA biosynthesis, the nature of the lipase will be discussed first.

Lipase

Support for the involvement of a lipolytic activity in the biosynthesis of JA came from the observations of Mueller and others (1993) and Conconi and others (1996a) that elicitation or wounding of plants caused rapid and transient changes in the lipid composition of plant cell membranes. A decrease in polar lipids concomitant with an increase of lysolipids, phosphatidic acid and free fatty acids (predominantly 18:3 and 18:2) correlated with the timing of JA accumulation. In analogy to the biosynthesis of animal prostaglandins which are structurally similar to JA (Mueller 1997), a PLA_2 was considered as a likely candidate for 18:3 release. Such an activity was detected in wounded tomato plants (Narvaez-Vasquez and others 1999) and its induction preceded oxylipin accumulation in tobacco mosaic virus-infected tobacco leaves (Dhondt and others 2000). An increase of PLA activity (likely a PLA_2) could also be detected in tobacco cells 2h after elicitation (Roy and others 1995) and in soybean after treatment with extracts of a pathogenic fungus (Chandra and others 1996).

On the other hand, inhibitors of animal PLA_2 did not prevent the accumulation of JA upon wounding of potato tuber tissue (Koda and Kikuta 1992). Furthermore in animal cells, the contribution of PLA_2 is consistent with the specific localization of

the prostaglandin precursor arachidonic acid (20:4) in the *sn-2* position of phospholipids. In the membranes of the chloroplast however, phospholipids are atypical and 18:3 is not commonly found in the *sn-2* position (Wallis and Browse 2002). Indeed, galactolipids account for more than 70% of the lipids in the inner membrane of the chloroplast envelope, whereas the contribution of phospholipids, mostly as phosphatidylcholine in the outer envelope, is less than 20% (Block and others 1983; Joyard and others 1991).

Characterization of the *dad1* mutant of *Arabidopsis* led to the identification of the lipase specifically involved in the biosynthesis of JA during male gametophyte development (Ishiguro and others 2001). The *dad1* mutant, like the other JA biosynthetic mutants (McConn and Browse 1996; Sanders and others 2000; Stintzi and Browse 2000; Park and others 2002; von Malek and others 2002) is male sterile, its JA content in flower buds is drastically reduced, and fertility could be restored by application of jasmonate to the floral organs. *In vitro*, DAD1 hydrolyzed galactolipids and triacylglycerol, but highest activity was observed for the *sn-1*-specific release of fatty acids from phospholipids. DAD1 was therefore defined as a PLA_1 (Ishiguro and others 2001). Consistent with its involvement in the initial steps of JA biosynthesis, DAD1 is targeted to the chloroplast. However, its precise localization within the chloroplast needs to be assessed to predict its physiological substrate(s). Defense responses are most likely not affected in *dad1* because a 100-fold induction in JA levels was observed in leaves of wounded plants (Ishiguro and others, unpublished results). This implies that lipolytic enzyme(s) other than DAD1, maybe (a) member(s) of the family of 12 *DAD1*-related genes (Beisson and others 2003), must be involved in the liberation of 18:3 upon wounding.

Finally, the contribution of a phospholipase D (PLD) in the wound-induced accumulation of JA was shown by antisense suppression of a PLD gene in *Arabidopsis* (Wang and others 2000). PLD however, does not release fatty acids as substrates for the octadecanoid pathway, and antisense-suppression of PLD is therefore not directly responsible for the observed reduction in JA. PLD may rather be indirectly involved by generating phosphatidic acid (PA) which might be the pivotal lipid messenger for the stimulation of lipid hydrolysis by other lipases (Wang 2004).

Lipoxygenase

Lipoxygenases (LOXs) catalyze the regio- and stereospecific dioxygenation of polyunsaturated fatty acids containing a (1Z,4Z)-pentadiene system to form fatty acid hydroperoxides (Rowley and others 1998; Brash 1999; Feussner and Wasternack 2002). Most LOXs are non-heme iron-containing dioxygenases which are widely distributed in animals and higher plants (Siedow 1991). Recently a secreted 15-LOX has been described in the opportunistic pathogen *Pseudomonas aeruginosa* as the first prokaryotic LOX (Vance and others 2004).

In plants, linoleic acid (18:2) and linolenic acid (18:3) are oxygenated at C-9 or C-13, and the corresponding (9*S*)-hydroperoxy or (13*S*)-hydroperoxy derivatives are further metabolized into a number of biologically active compounds (oxylipins). In the Vick and Zimmerman pathway, 13(*S*)-hydroperoxy linole(n)ic acid produced by 13-LOX from 18:3 (18:2) is the substrate for jasmonate biosynthesis (Figure 1) (Vick and Zimmerman 1983; Siedow 1991; Feussner and Wasternack 2002)

The majority of plant LOXs appears to act on free 18:2 and 18:3 despite the poor solubility in aqueous media at typical physiological pH values of 7.0-7.4, (Siedow 1991; Brash 1999; Feussner and Wasternack 2002). This would be consistent with the substantial increase of free 18:2 and 18:3 observed after wounding in tomato plants (Conconi and others 1996a). Free 18:2 and 18:3 and even free fatty acid hydroperoxides were also found to accumulate in barley after treatment with MeJA suggesting free fatty acids to be the LOX substrates in oxylipin biosynthesis and, correspondingly, a 13-LOX was localized within the plastid stroma (Bachmann and others 2002). Fatty acids esterified to membrane lipids, however, have to be considered as alternative substrates (Figure 3). A plant 13-LOX has been shown to act on polyunsaturated fatty acids esterified to phospholipids and triglycerides (reviewed by Feussner and Wasternack 2002). Furthermore, galactolipids carrying dinor-oxophytodienoic acid (dnOPDA) in the *sn1* and *sn2* positions have recently been discovered and may be the result of *in situ* formation of lipid hydroperoxides (Figure 3) (Stelmach and others 2001; Hisamatsu and others 2003).

Higher plants contain multiple LOXs comprising at least eight isoforms in soybean and six genes in *Arabidopsis* which have been implicated in many aspects of plant growth and development (for review see Brash 1999; Feussner and others 2001; Feussner and Wasternack 2002). Of particular interest in this article is the function of 13-LOXs as part of the octadecanoid pathway for jasmonate biosynthesis, which has been addressed predominantly in the context of plant defense and senescence, two processes well known to involve jasmonate signaling molecules. At least three of the

six LOXs in *Arabidopsis* (LOX2, 3, 4) appear to contribute to jasmonate biosynthesis in defense and senescence. Consistent with the localization of the preceding and subsequent steps of the octadecanoid pathway, these LOXs are targeted to plastids. The stroma-localized LOX2 was shown to be required for the wound-induced accumulation of JA and the expression of defense genes in *Arabidopsis* leaves (Bell and Mullet 1993; Bell and others 1995). The senescence program, on the other hand involves the induction of LOX3 and LOX4 as well as the cytosolic LOX1, while LOX2 is downregulated (Melan and others 1993; Creelman and Mullet 1997; He and others 2002).

Several studies focused on the 13-LOX pathway in the wound response in *Solanaceae.* Antisense depletion of a specific 13-LOX isoform in potato compromised the wound-induced expression of defense genes and increased susceptibility to insect pests (Royo and others 1999). In *Nicotiana attenuata*, the depletion of a 13-LOX (*Na*LOX3) impaired the wound- and herbivory-induced accumulation of JA and expression of defense genes as well as herbivore resistance (Halitschke and Baldwin 2003; Halitschke and Baldwin, this issue). Also in their natural habitat, these plants were found to be more vulnerable to specialist herbivores and, furthermore, attracted novel herbivore species which fed and reproduced successfully (Kessler and others 2004). The data indicate that *Na*LOX3 supplies fatty acid hydroperoxides as substrates for jasmonate synthesis in herbivore defense (Halitschke and Baldwin 2003). In addition to wound signaling, LOX-deficiency was also shown to affect pathogen defense in tobacco (Veronesi and others 1996; Rance and others 1998).

Allene Oxide Synthase

The fatty acid hydroperoxides generated by 9-LOX and 13-LOX from polyunsaturated fatty acids are precursors for a vast array of different oxylipins. The Vick and Zimmerman pathway (Figure 1) constitutes but one of seven metabolic fates for hydroperoxy fatty acids (Figure 2). It channels the 13-hydroperoxides of 18:3 and 18:2 into jasmonate biosynthesis and the committed step of this pathway is catalyzed by allene oxide synthase (AOS; Vick and Zimmerman 1987). AOS catalyzes the dehydration of the hydroperoxides to form an unstable epoxide which either spontaneously hydrolyzes to a mixture of α- and γ-ketols (80% and 10%, respectively) and racemic *cis*-12-oxophytodienoic acid (OPDA, 10%) or, in a concerted action with allene oxide cyclase (AOC), is converted into enantiomerically pure 9*S*,13*S*-OPDA (*cis*(+)-OPDA; Hamberg and Fahlstadius 1990; Laudert and others 1997).

AOS belongs to branch A of the CYP74 family of soluble cytochrome P450s which is characterized by independence of molecular oxygen and NADPH, and low affinity for CO. The CYP74 enzymes use a hydroperoxide group as both the oxygen donor and as a source for reducing equivalents (Song and others 1993; Pan and others 1995; Laudert and others 1996; Howe and Schilmiller 2002). Other CYP74 enzymes include hydroperoxide lyases (HPLs) in branches B and C and divinyl ether synthases (DESs) in branch D which direct fatty acid hydroperoxides each to a different class of oxylipins (Feussner and Wasternack 2002; Howe and Schilmiller 2002). Interestingly, the P450s in the CYP74A and B subfamilies appear to be specific for 13-hydroperoxides (13-LOX products) whereas those in subfamilies C and D metabolize 9-hydroperoxides (9-LOX products). The relative specificities of these enzymes for either the 9- or the 13-hydroperoxides supports the concept that oxylipin metabolism is organized into discrete 9-LOX and 13-LOX pathways each of which splits into several CYP74-dependent branches (Howe and Schilmiller 2002).

The concept is supported by the recent characterization of diverse 9-hydroperoxide-derived oxylipins with roles in plant defense and development (Grechkin and others 1995; Weber and others 1999; Yokoyama and others 2000; Gobel and others 2001; Kolomiets and others 2001; Stumpe and others 2001). Of particular interest in this context, is the existence of a 9-LOX/AOS pathway which has been demonstrated in potato (Hamberg 2000) and tulip bulbs (Grechkin and others 2000). Structural isomers of OPDA, that is, 10-oxo-11-phytoenoic acid (10-OPEA) and 10-oxo-11,15-phytodienoic acid (10-OPDA) were identified as metabolites of this pathway in potato (Hamberg 2000) and AOS3 from tomato (a CYP74C) was cloned and shown to be about 10-fold more active against 9-hydroperoxides of linolenic and linoleic acids than the corresponding 13-isomers (Itoh and others 2002). The physiological function of the 9-LOX/AOS pathway is still unknown but given the structural similarity of its products to those of the 13-LOX/AOS pathway, a role in signaling is anticipated.

The AOS committed to JA synthesis was first purified and cloned from flax seeds (Song and Brash 1991; Song and others 1993) and subsequently identified in guayule (Pan and others 1995), *Arabidopsis* (Laudert and others 1996), tomato (Howe and others 2000; Sivasankar and others 2000), and barley (Maucher and others 2000;

Maucher and others 2004). In *Arabidopsis* the enzyme is encoded by just one gene as confirmed by the phenotype of single gene, loss-of-function mutants: AOS-deficient plants were male-sterile, failed to accumulate JA after wounding, and were compromised in the induction of wound- and JA-dependent gene expression (Park and others 2002; von Malek and others 2002), traits shared with other jasmonate biosynthesis and perception mutants (Feys and others 1994; McConn and others 1997; Sanders and others 2000; Stintzi and others 2001).

AOS activity has been localized to spinach chloroplasts (Vick and Zimmerman 1987). The presence of amino-terminal transit peptides also indicates plastidial localization for the enzymes from flax (Song and others 1993), *Arabidopsis* (Laudert and others 1996), and tomato (Sivasankar and others 2000), and targeting to the stromal side of the inner envelope membrane has been confirmed for tomato AOS (Froehlich and others 2001). The two isoforms of barley AOS appear to be targeted to plastids despite the lack of typical transit peptides (Maucher and others 2000) leaving the guayule AOS in rubber particles as the so far only exception from plastid localization (Pan and others 1995).

The current model of oxylipin synthesis which depicts a number of different enzymes, each acting on hydroperoxide substrates but dedicated to the synthesis of a specific class of oxylipins, suggests that the activity of these enzymes may control the flux into the respective metabolic pathway (Figure 2). In fact, a redirection of LOX products into the hydroperoxide reductase branch has been observed in barley upon treatment with salicylate (Weichert and others 1999) whereas the hydroperoxide reductase and lyase branches were preferentially activated upon jasmonate treatment (Bachmann and others 2002). Consistent with such a model, the overexpression of flax AOS in transgenic potato plants led to a 6-12 fold increase in constitutive levels of JA (Harms and others 1995). In contrast, the overexpression of *Arabidopsis* AOS in either *Arabidopsis* or tobacco did not alter basal levels of JA. The wound-induced accumulation of JA, however, was more pronounced and the JA maximum was reached significantly earlier in the AOS transgenics as compared to wounded control plants (Wang and others 1999; Laudert and others 2000; Park and others 2002). The latter results would suggest that substrate availability, controlled by upstream enzymes, determines JA levels at least in unwounded plants. Only upon wounding, when an abundance of hydroperoxide substrates is generated, is AOS activity limiting the flux into the JA-biosynthetic pathway (Wang and others 1999; Laudert and others 2000; Park and others 2002).

The expression of AOS has in fact been shown to be wound-inducible in many plant species and was found to be upregulated by JA and OPDA (Harms and others 1998; Laudert and Weiler 1998; Maucher and others 2000; Sivasankar and others 2000; Strassner and others 2002), which supports the existence of a positive feed-back loop to enhance the flux into the biosynthetic pathway and the accumulation of jasmonates after wounding. Evidence for such an amplification loop was provided by the *opr3* mutant in which the wound-induced levels of OPDA were reduced as compared to wild-type plants, likely due to the lack of JA in the mutant and therefore the lack of auto-amplification (Stintzi and others 2001; Stenzel and others 2003b). The feed-back loop appears to be activated in the *cas1* mutant of *Arabidopsis* explaining its molecular phenotype, which includes enhanced levels of AOS mRNA and protein, OPDA and JA, and constitutive expression of JA-dependent genes (Kubigsteltig and Weiler 2003).

A detailed analysis of AOS expression was performed in transgenic plants carrying a promoter *AOS::uidA* reporter gene. In both tobacco and *Arabidopsis*, the local and systemic activation of gene expression from the AOS promoter was observed after wounding (Kubigsteltig and others 1999). Two hours after wounding, GUS activity was observed in the main veins and petioles of *Arabidopsis* and tobacco leaves and 24 hours after wounding, the whole shoot had reacted uniformly. This is in contrast to findings in tomato, where the systemic induction of AOS (and other octadecanoid pathway genes) was much attenuated as compared to the expression in the wounded leaf (Howe and others 2000; Strassner and others 2002). Following the localized application of OPDA, JA, and the octadecanoid analog coronatin, the induction of AOS promoter activity was observed only in the treated leaves thus arguing against a role for jasmonates as signal molecules in the systemic response (Kubigsteltig and others 1999). Recent grafting experiments of the JA responsive *VSP*-promoter::*LUC* transgenic plants with the JA-insensitive *coi1*, the *dad1* and the *opr3* mutants, however, indicate that the systemic response requires JA but not JA synthesis in the systemic leaves (Hawkes and Turner 2004). These results confirm previous findings in tomato, where the systemic response to wounding was shown to depend on jasmonate synthesis and jasmonate perception in local and systemic tissues, respectively (Li and others 2000; Strassner and others 2002). The analysis of GUS expression from

the AOS promoter also revealed developmental regulation. The reporter was found to be expressed in early stages of carpel development, at the base of elongated anther filaments, and in maturing pollen (Kubigsteltig and others 1999). Expression in the developing flower is consistent with the presence of jasmonates in flower organs (Hause and others 2000) and the requirement for JA in anther development (Sanders and others 2000; Stintzi and Browse 2000).

Allene Oxide Cyclase

In 1981, Vick and Zimmerman (1981) postulated the existence of a hydroperoxide cyclase for the conversion of 13(*S*)-HPOT into OPDA, and the allene oxide 12,13(*S*)-epoxylinolenic acid was shown to serve as the immediate precursor of OPDA in plants (Baertschi and others 1988; Crombie and Morgan 1988; Hamberg and Hughes 1988). In 1990, Hamberg and Fahlstadius (1990) showed that the reaction is catalyzed by allene oxide cyclase (AOC), a soluble enzyme in corn with a molecular mass of about 45 kDa. AOC was subsequently purified as a dimer of 47 kDa from maize kernels (Ziegler and others 1997) and characterized with respect to its substrate specificity: the enzyme accepted 12,13(*S*)-epoxylinolenic acid but not 12,13(*S*)-epoxylinoleic acid, as a substrate (Ziegler and others 1999). This is in contrast to AOS which produces both allene oxides from the respective 13(*S*)-hydroperoxy fatty acids (18:3 and 18:2, respectively). It thus appears that AOC confers additional specificity to the octadecanoid biosynthetic pathway.

An interesting aspect of the AOC reaction is the apparent competition between the spontaneous decomposition of its substrate, the unstable allene oxide, to form α- and γ- ketols and racemic *cis*-OPDA and the enzyme-catalyzed formation of optically pure (9*S*,13*S*)-OPDA (that is, *cis*(+)-OPDA), the first pathway intermediate having the characteristic pentacyclic ring structure of jasmonates (Figure 1). The extremely short half-life of allene oxides ($t_{1/2}$ less than 30 s in water), the optical purity of natural OPDA, and the absence of detectable levels of α- and γ-ketols in plant tissues *in vivo* (Laudert and others 1997; Stelmach and others 1998; Weiler and others 1998), suggest a tight coupling of the AOS and AOC reactions possibly in a synthase-cyclase complex (Ziegler and others 1999). Coupling of the two reactions is also observed *in vitro:* AOC from potato or recombinant *Arabidopsis* AOC2 in combination with recombinant *Arabidopsis* AOS resulted in the production of highly asymmetrical *cis*-OPDA consisting nearly exclusively of the (95,13*S*)-enantiomer (Laudert and others 1997; P. Zerbe and F. Schaller, unpublished).

AOC has been cloned as a single-copy gene from tomato (Ziegler and others 2000) and barley (Maucher and others 2004) and as a small gene family (*AOC1-4*) from *Arabidopsis* (Stenzel and others 2003a). Inspection of the N-termini of the cloned AOCs revealed the presence of transit peptides for plastid targeting, and localization in chloroplasts was confirmed immunohistochemically (Ziegler and others 2000; Stenzel and others 2003a) and by transient expression of AOC1-4/GFP fusion proteins (P. Zerbe and F. Schaller unpublished).

In vegetative tissues of tomato plants, the expression of AOC was found to be confined to vascular bundles where it was induced by JA and the oligopeptide wound signal systemin leading to an increased production of JA in the main vein rather than the leaf lamina (Hause and others 2003a; Stenzel and others 2003a). The precursor protein of systemin, prosystemin, is also expressed in vascular bundles and is induced by wounding and JA as well (Jacinto and others 1997; Narvaez-Vasquez and Ryan 2004). These findings are consistent with a model of wound signaling according to which tissue injury leads to the release/formation of small amounts of JA and systemin. JA would enhance the production of systemin which, in turn, further stimulates the octadecanoid pathway resulting in an amplification loop for enhanced production of jasmonates in the vascular system where they may act as the mobile signal for the systemic induction of defense proteins (Ryan and Moura 2002; Stenzel and others 2003a). This scenario is supported by studies showing that (i) the production of systemin and JA in the wounded leaf is necessary for systemic wound signaling, whereas in systemic tissues, only JA perception but no synthesis is required (Li and others 2002) and (ii) wounding of tomato plants leads to the induction of octadecanoid pathway enzymes for enhanced production of jasmonates in the wounded, but not in unwounded systemic leaves (Strassner and others 2002).

Constitutive overexpression of AOC (10-fold increase in activity) did not lead to an increase in basel levels of OPDA and JA in leaf tissue (Stenzel and others 2003a). Both compounds, however, accumulated to higher levels after wounding in the AOC overexpressors (or AOS overexpressors; see previous paragraph) as compared to the wild type plants. Apparently, the production of jasmonates is limited by the availability of substrates (free 18:2, 18:3, and the respective hydroperoxides) which are enhanced in leaves after wounding. Interestingly, however, this does not seem to be the case in flowers: Con-

stitutive overexpression of AOC led to a dramatic increase in jasmonate content in flowers and to substantial changes in the relative concentrations of individual jasmonates (the oxylipin signature; Hause and others 2000) between different flower organs (Miersch and others 2004). Thus, jasmonate biosynthesis appears to be differentially regulated in an organ-specific manner.

12 Oxophytodienoic Acid Reductase

Analyzing the metabolism of ^{18}O-labelled OPDA in several plant tissues, Vick and Zimmerman identified 3-oxo-2-(2′(Z)-pentenyl)-cyclopentane-1-octanoic acid (OPC-8:0) as one of the labelled metabolites (Vick and Zimmerman 1984). The enzyme catalyzing the reduction of the C=C double bond of the conjugated enone moiety was called 12-oxophytodienoic acid reductase (OPR). The first purification of OPR activity from Rock Harlequin (*Corydalis sempervirens*) employed an assay with racemic *cis*-OPDA (9*S*,13*S*/cis(+)- and 9*R*, 13*R*/*cis*(-)-OPDA) as the substrate (Schaller and Weiler 1997a). Further characterization of the purified enzyme revealed specificity for the *cis*(-)-enantiomer which may arise *in planta* from partial uncoupling of the AOS/AOC reactions but is not a precursor of JA in the octadecanoid pathway (Schaller and others 1998). The finding indicated that (an) additional OPR isoform(s) must exist for the reduction of *cis*(+)-OPDA. Indeed, a small OPR gene family exists and has been characterized in several plant species including two OPRs from Rock Harlequin (Schaller and others 1998), five *OPR* genes three of which have been characterized in *Arabidopsis* (Schaller and Weiler 1997b; Biesgen and Weiler 1999; Costa and others 2000; Mussig and others 2000; Schaller and others 2000), six OPRs from pea (Ishiga and others 2002; Matsui and others 2004), and three OPR isoforms from tomato (Straβner and others 1999; Strassner and others 2002). Thirteen OPRs are encoded in the rice genome, but only one of the enzymes has been characterized so far (Agrawal and others 2003, 2004; Sobajima and others 2003). All these enzymes catalyze the reduction of α,β-unsaturated carbonyls (conjugated enones) in a wide variety of substrates including OPDA, with NADPH as the reducing agent. For the OPR isoform involved in the octadecanoid pathway a preference for *cis*(+)-OPDA, the naturally occurring precursor of JA, would be expected and has been shown for OPRII from Rock Harlequin (Schaller and others 1998), OPR3 from *Arabidopsis* (Schaller and others 2000), and *Le*OPR3 from tomato (Strassner and others 2002). Thus, OPR3 rather than OPR1 and OPR2 appears to be the enzyme involved in JA biosynthesis in *Arabidopsis* and tomato. The same conclusion was reached independently using a genetic approach, namely the characterization of the *opr3(dde1)* mutant in *Arabidopsis*. In *opr3*, a single gene knockout leads to JA-deficiency and male sterility, indicating that OPR3 can not be substituted by other OPR isoforms in JA biosynthesis (Sanders and others 2000; Stintzi and Browse 2000). Hence, there is no direct involvement of OPRs 1 and 2 in JA biosynthesis and their function remains obscure. Considering the broad substrate specificity of *Le*OPR1 and *Arabidopsis* OPR1 for α,β-unsaturated carbonyls *in vitro* (Straβner and others 1999; F. Schaller, unpublished), potential *in vivo* substrates include many physiologically relevant compounds.

Plant OPRs are closely related to Otto Warburg's Old Yellow Enzyme (OYE), a flavoprotein enone reductase initially isolated from brewer's bottom yeast, which was the first enzyme shown to possess a flavin cofactor (Warburg and Christian 1932, 1933). There is substantial sequence conservation between the yeast and the plant enzymes including two of the three amino acids involved in substrate binding that is, Tyr376 and His192 (numbering refers to the OYE sequence), whereas Asp195 is replaced by His in most of the plant sequences. As for OPRs 1 and 2, the physiological substrate(s) and function(s) of OYE are still elusive, despite extensive biochemical and spectroscopic characterization (Massey and Schopfer 1986; Stott and others 1993; Vaz and others 1995).

Yeast OYEs form charge transfer (CT) complexes with a variety of aromatic and heteroaromatic compounds carrying an ionizable hydroxyl group. Formation of the CT complex results in a long wavelength transition in the absorbance spectra. The absorbance maximum was shown to correlate with the ionization potential of the phenolic ligand suggesting that the bound phenolate and the oxidized flavin act as the electron donor and acceptors, respectively (Abramovitz and Massey 1976; Massey and others 1984). The formation of CT complexes with a series of phenolic ligands was also observed for *Le*OPR1 (Straβner and others 1999; Zheng and others 2001) and OPR3s from tomato and *Arabidopsis* (A. Schaller, F. Schaller and P. Macheroux unpublished) providing further biochemical evidence for the close relationship between yeast OYEs and their plant homologs.

Exploiting the known crystal structure of OYE (Fox and Karplus 1994) and the similarity of the yeast and the plant enzymes, the X-ray structure of OPR1 from tomato was solved by molecular replacement (Breithaupt and others 2001). *Le*OPR1

crystallizes as a monomer and folds into a $(\beta)_8$ barrel with an overall structure similar to OYE. The structure of the enzyme substrate complex (*cis*(-)-OPDA/*Le*OPR1) confirms the mechanism of substrate reduction that had been proposed for the OYE family on the basis of mechanistic studies. Briefly, hydrogen bonding of the carbonyl oxygen to His187 and His197 leads to a polarization of the olefinic bond facilitating hydride transfer from the flavin N(5) to the substrate Cβ. The substrate Cα is then protonated from the opposite side by Tyr 192 (Breithaupt and others 2001). The mode of substrate binding and the active site structure in *Le*OPR1 clearly indicate α,β-unsaturated carbonyl compounds as substrates of the OYE family. Furthermore, extensive van der Waals contacts were observed between the alkyl side chain of *cis*(-)-OPDA and a tunnel formed by the Lβ3 loop of *Le*OPR1 supporting *cis*(-)-OPDA or related oxylipins as the physiological substrates (Breithaupt and others 2001).

Specific binding of *cis*(-)-OPDA by Lβ3 results in the stereoselectivity of *Le*OPR1 (Breithaupt and others 2001). Indeed, in the *Le*OPR3 structure, considerable differences for the corresponding loop and a more open active site cavity have been observed. These differences explain the more relaxed specificity of *Le*OPR3, allowing reduction of both the *cis*(+) and the *cis*(-) enantiomers of OPDA (C. Breithaupt, J. Strassner, T. Clausen, P. Macheroux and A. Schaller unpublished). *Le*OPR3 was found to crystallize as a homodimer. The *Le*OPRS dimer appears to be inactive because each subunit specifically blocks the active site of the other (C. Breithaupt, J. Strassner, T. Clausen, P. Macheroux and A. Schaller unpublished). Hence, the regulation of OPR3 activity by control of the dimer/monomer equilibrium appears feasible. Whether or not this potential mechanism is relevant for the regulation of enzyme activity *in vivo* is still unknown.

Understanding the regulation of OPR activity is of fundamental interest because OPDA appears to have signaling properties that are distinct from JA. Indeed, β-oxidation was shown not to be required for phytoalexin induction in cell cultures indicating that OPDA is active *per se* in this system (Blechert and others 1995; Parchmann and others 1997), and different signaling properties were observed for JA and OPDA in stressed barley leaves (Kramell and others 2000). Furthermore, OPDA rather than JA seems to be the endogenous signal transducer in mechanotransduction (Weiler and others 1993; Weiler and others 1994; Stelmach and others 1998). In tendrils of *Bryonia dioica*, there is a strong and transient increase in OPDA levels after mechanical stimulation while JA levels remain unaffected (Blechert and others 1999). Thus OPR3 may play a decisive role in jasmonate signaling in that the enzyme potentially controls the metabolite flow from the biologically active cyclopentenone OPDA to the cyclopentanones including JA. Such a role for OPR3 and differential signaling activities of OPDA and JA have been demonstrated by genetic means in *Arabidopsis*. The *Arabidopsis opr3* mutant is male sterile as a result of shortened anther filaments, delayed dehiscence of the anther locule, and reduced pollen viability. All these effects could be alleviated by external application of JA but not by OPDA providing clear evidence for a role of JA rather than OPDA in the respective developmental processes (Sanders and others 2000; Stintzi and Browse 2000). JA-dependent defense responses, on the other hand were not impaired in *opr3*. The mutant was found to be fully resistant to larvae of *Bradysia impatiens* and the fungus *Alternaria brassicicola* suggesting that OPDA can substitute for JA in the activation of defense responses. Indeed, OPDA was found to induce many JA-dependent genes in the *opr3* mutant while a subset of defense-related genes was actually activated by OPDA but not by JA, indicating overlapping as well as distinct signaling functions (Stintzi and Browse 2000; Stintzi and others 2001; Mithöfer and others this issue).

So far, the regulation of OPRs has been shown only at the level of gene expression. Although the expression of *OPR3* was stimulated under the very same conditions that are known to activate the jasmonate signaling pathway (Farmer and Ryan 1990; Falkenstein and others 1991; Conconi and others 1996b; Stelmach and others 1998; Strassner and others 2002), *OPR1* and *2* genes were activated by diverse forms of environmental stress (Iuchi and others 1996; Biesgen and Weiler 1999; Reymond and others 2000; He and Gan 2001; Ishiga and others 2002; Agrawal and others 2003). A common denominator for these different forms of stress is the production of α,β-unsaturated carbonyls with cytotoxic and/or regulatory activities resulting from the accumulation of reactive oxygen species (Deighton and others 1999; Vollenweider and others 2000; Muckenschnabel and others 2001; Almeras and others 2003). Induction of OPRs 1 and 2 under such conditions may indicate a role in the detoxification of these reactive molecules. In fact, the specific induction of *OPR1* gene expression under conditions of oxidative stress has been observed in *Arabidopsis* (C. Ochsenbein and K. Apel, pers. communication).

In tomato plants, *OPR3* but not *OPR1* and *2* transcript levels increased concomitant with the accumulation of OPDA and JA in wounded leaves.

As compared to the transient JA peak, OPDA accumulation was more sustained supporting a regulatory role for OPR3 in controlling the relative levels of OPDA and JA, that is, two signaling molecules with potentially different acivities (Strassner and others 2002).

In *Arabidopsis* flowers, as demonstrated by all JA biosynthetic (McConn and Browse 1996; Sanders and others 2000; Stintzi and Browse 2000; Ishiguro and others 2001; Park and others 2002; von Malek and others 2002) and the *coi1* signaling mutants (Feys 1994), JA coordinates the elongation of the anther filaments, the opening of the stomium at anthesis, and the maturation of the trinucleate pollen grains. The male-sterile phenotype in these plants is controlled by the genotype of the sporophytic tissue, and all defects can be corrected by application of JA to flower buds of the JA-deficient plants. Only flower buds in stage 12 of floral development (see Smyth and others 1990) responded to JA, which corresponds to the final stage before the bud opens and anther dehiscence occurs, and encompasses the second mitotic pollen division. Expression of *OPR3* (*DDE1*) in developing flowers was analyzed by *in situ* hybridization, and the transcript was detected throughout premeiotic anthers, and specifically in the pistil, petals, and the base of the stamen filaments long before and until the initiation of the anther dehiscence program (Sanders and others 2000). These results suggest that the gene is not expressed, and therefore JA not produced in those cells of the anther that play a role in dehiscence or maturation of the pollen grains. If produced within the anther at all, then JA must originate from synthesis during the early stages of development. Alternatively, the JA signal that is needed in stage 12 in the anther could be produced as a result of *OPR3* expression in the pistil, petals and/or the anther filaments. Indeed, loss of the specific expression of DAD1 in anther filaments is sufficient to create the male-sterile mutant phenotype (Ishiguro and others 2001).

In addition to regulation at the transcript level, OPR3 may be regulated by protein stability/degradation, dimer dissociation (see above), and substrate availability. The substrate OPDA is produced by the consecutive action of LOX, AOS, and AOC in plastids. For OPR3, however localization in the peroxisome was proposed based on the presence of the respective targeting signal at the extreme carboxy terminus (Stintzi and Browse 2000) and targeting to the peroxisomes has later been confirmed for OPR3 s from both *Arabidopsis* and tomato (Strassner and others 2002). Because OPR3 is the only OPR in the octadecanoid pathway, the synthesis of JA requires transport of OPDA from the plastids to the peroxisomes for further metabolism by OPR3 and β-oxidation enzymes. The molecular basis of OPDA transport is still unknown. However, whatever the transport mechanism is, it has the potential to control the relative concentrations of cyclopentenones and cyclopentanones, that is, jasmonates with different signaling properties.

β-Oxidation

Investigating the metabolism of ^{18}O-labelled OPDA, OPC8:0 and 15,16-dihydro-OPC8:0, Vick and Zimmermann observed the formation of JA and 9,10-dihydro-JA, respectively. Furthermore, two labeled C16 and C14 intermediates were found with the acyl side chain shortened by two and four carbons, respectively. These intermediates are indicative of three cycles of β-oxidation as the terminal steps in JA biosynthesis (Vick and Zimmerman 1983, 1984) Although the compartmentalization of these β-oxidation steps has not been addressed specifically yet, they are likely to be localized in the peroxisomes which are the main site of β-oxidation in plants (Gerhardt 1983; Vick and Zimmerman 1984). This conclusion is supported by the peroxisomal localization of the preceding step in jasmonate biosynthesis, the one catalyzed by OPR3 (Strassner and others 2002).

Peroxisomal β-oxidation is catalyzed by three proteins (i) acyl-CoA oxidase (ACX), (ii) the multifunctional protein (MFP; exhibiting 2-*trans*-enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, D-3-hydroxyacyl-CoA epimerase and Δ^3,Δ^2-enoyl-CoA isomerase activities), and (iii) L-3-ketoacyl-CoA thiolase (KAT). Together these proteins catalyze the repeated cleavage of acetate units from acyl-CoAs. Each of the three functions is encoded by a small gene family in *Arabidopsis.* The MFPs have broad overlapping substrate specificities, which has also been shown for (at least some of) the KATs (Behrends and others 1988; Preisig-Müller and others 1994; Germain and others 2001, and references therein). The four *Arabidopsis* ACXs, on the other hand, are quite distinct with respect to their substrate specificities, but together they accomodate the full range of acyl-CoA chain lengths that exist *in vivo* (Eastmond and others 2000; Rylott and others 2003). The limited specificity of peroxisomal β-oxidation is evident also from the finding that the proherbicide 2,4-dichlorophenoxybutyric acid and the prohormone indole butyric acid are β-oxidized to 2,4-D and IAA, respectively (Hayashi and others 1998; Zolman and others 2000). Consequently, the CoA-ester of OPC8:0 would be expected to be a

substrate of regular peroxisomal β-oxidation. However, mutants disrupted in each of the three steps have been described (Hayashi and others 1998; Richmond and Bleecker 1999; Eastmond and others 2000; Rylott and others 2003) but no defect in JA biosynthesis has been reported. Although this could be explained by functional redundancy of the different isoforms, alternatives to regular peroxisomal β-oxidation should still be considered.

The apparent lack of substrate specificity in peroxisomal β-oxidation is inconsistent with data showing that specificity does in fact exist with respect to jasmonate biosynthesis: Although side chain-shortened intermediates were observed for the conversion of OPC8:0 to JA in wild-type plants, such intermediates and 4,5-didehydro-JA were never observed in the *opr3* mutant (Stintzi and others 2001; A. Stintzi and H. Weber unpublished), indicating that OPC8:0 but not OPDA is subject to β-oxidation. Considering the apparent promiscuity of the β-oxidation enzymes proper, the required specificity may rather reside in either the transport or the activation step of OPDA

Activation of the fatty acid carboxyl group and formation of the acyl-CoA substrates for β-oxidation may occur in the cytosol concomitant with import into peroxisomes by PXA1, an ABC-type transporter in the peroxisomal membrane (Zolman and others 2001b). Alternatively, the free fatty acids may be delivered to the peroxisomes by PXA1 and subsequently activated by peroxisomal acyl-CoA synthetases (ACSs; Fulda and others 2004). In case of jasmonate biosynthesis, OPC8:0 is generated from OPDA within peroxisomes by action of OPR3 and, therefore, is likely to be activated by a peroxisomal ACS. ACSs are encoded by a large gene family in *Arabidopsis* and differences in substrate specificity have been demonstrated for individual enzymes (Shockey and others 2003; Schnurr and others 2004). An ACS specific for OPC8:0 remains to be identified, but could explain the apparent exclusion of OPDA from β-oxidation. Alternatively, transport of OPDA from the plastids to the peroxisomes and thus the access to β-oxidation may be inhibited in *opr3* in the absence of JA.

During β-oxidation, JA should be produced as a CoA-ester implying the existence of a thioesterase/acyl-CoA hydrolase to release free JA. Likewise, the detection of OPC6:0 and OPC4:0 (Vick and Zimmerman 1984; H. Weber and A. Stintzi unpublished) implies the release of these pathway intermediates from the respective CoA-esters. Two peroxisomal acyl-CoA thioesterases have been characterized in *Arabidopsis*: ACH2 which exhibits high activity toward medium and long chain acyl-CoAs (Tilton and others 2004), and CHY1 which hydrolyzes β-hydroxyisobutyryl-CoA in valine catabolism (Zolman and others 2001a). Whether or not these enzymes can act on CoA esters of JA, OPC6:0, or OPC4:0 is not known.

Alternative Pathways for the Biosynthesis of Jasmonates

The Vick and Zimmermann pathway starting with free linolenic acid (18:3) as the substrate for JA biosynthesis is not the only possible route to jasmonates and several variations on that theme should be considered (Figure 3).

Linoleic acid (18:2) could be an alternative substrate and if it were metabolized by all the enzymes of the Vick and Zimmermann pathway would yield 9,10-dihydro-JA (DHJA), a derivative of JA in which the double bond of the pentenyl side chain is reduced. Occurrence and bioactivity of DHJA have been shown *in vivo* (Ueda and others 1981; Miersch and others 1989; Blechert and others 1995; Gundlach and Zenk 1998). Other studies, however, failed to detect DH-jasmonates and, depending on the bioassay employed, DHJA was found to be inactive suggesting that the pathway may not be operating in all species (referenced in Gundlach and Zenk 1998). The bottle-neck appears to be allene oxide cyclase which is the only enzyme of the pathway able to discriminate between 18:3 and 18:2-derived pathway intermediates (Gundlach and Zenk 1998). As mentioned earlier, the 18:2-derived allene oxide was found not to be a substrate of corn AOC (Ziegler and others 1999). In linseed, however, the formation of DH-OPDA from 18:2 was observed (Gundlach and Zenk 1998). AOCs from different species may thus differ in substrate specificity or, alternatively, DH-OPDA (and further DH-jasmonates) may result from the spontaneous cyclization of the 18:2-derived allene oxide.

A 16-carbon cyclopentenoic acid analog of OPDA, dinor-oxophytodienoic acid (dnOPDA), was identified in leaf extracts of *Arabidopsis* and potato plants and a dramatic increase upon wounding suggested a role in wound signaling (Weber and others 1997). DnOPDA could potentially arise from OPDA by β-oxidation of the octanoate side chain, or be derived from hexadecatrienoic acid (16:3) via the Vick and Zimmerman pathway (Figure 3). The *Arabidopsis fad5* mutant is incapable of synthesizing 16:3 and is devoid of dnOPDA thus confirming 16:3 as the precursor of dnOPDA (Weber and others 1997).

A role for dnOPDA in wound signaling is also supported by the *spr2* (*LeFad7*) mutant in tomato which is compromised in plastid fatty acid desatu-

ration. In *spr2*, plastid 18:3 content is reduced to less than 10% of the wild-type level, whereas 16:3 is undetectable. Concomitant with the reduction in trienoic fatty acids, an increase in the corresponding dienoic acids (18:2, 16:2) was observed (Li and others 2003). The mutant is severely compromised in both the local and the systemic wound response, the production of the systemic wound signal, and shows increased susceptibility to insect herbivores (Li and others 2002; Li and others 2003; Howe this issue). Apparently, jasmonates potentially derived from the residual 18:3 or from the elevated 18:2 are not sufficient to activate the wound response in tomato. The 16:3 deficiency of *spr2*, on the other hand, points toward such a role for 16:3-derived jasmonates, namely dnOPDA.

Another interesting finding is the discovery of OPDA and/or dnOPDA in plastid lipids. Indeed, 80% of the OPDA (E. W. Weiler pers. communication) appears to be present in esterified form in the *sn1* position of a monogalactosyl diacylglyceride (MGDG-O). In MGDG-O, the *sn2* position is occupied by 16:3, indicating synthesis via the ''prokaryotic pathway'' of lipid formation (Stelmach and others 2001; Kelly and others 2003). Related but less abundant lipids with OPDA and dnOPDA or two molecules of OPDA in *sn1* and *sn2* were identified and named Arabidopside 1 and 2, respectively (Hisamatsu and others 2003). It is presently unclear whether lipid-bound (dn)OPDA is synthesized *in situ* from MGDGs or, alternatively, in the plastid stroma from free 18:3 (16:3) and subsequently activated and incorporated into the lipid fraction (Figure 3). The latter possibility is supported by substantial amounts of free 18:3 and 18:2 that were detected in tomato leaves after wounding (Conconi and others 1996a) and of free fatty acid hydroperoxides accumulating in senescing barley leaves (Bachmann and others 2002). On the other hand, plant lipoxygenases are known to act on both free and esterified fatty acids (Feussner and others 1995; Feussner and others 1997; Brash 1999; Feussner and Wasternack 2002), and *Arabidopsis* leaves (Stenzel and others 2003b) and tomato flower organs (Miersch and others 2004) contain much more esterified than free fatty acid hydroperoxides. Furthermore, it has been shown that enzymes of the jasmonate biosynthetic pathway, including LOX and AOS, act on *N*-acyl(ethanol)amines to yield, among other products, 12-oxo-*N*-phytodienoylamines (van der Stelt and others 2000). The synthesis of (dn)OPDA in esterified form thus seems feasible and free (dn)OPDA may be generated by hydrolysis of membrane lipids. In fact, such a membrane-associated hydrolytic activity appears to be present in *Arabidopsis* (Stelmach and others 2001).

The available evidence indicates that OPDA, a highly active jasmonate, is formed constitutively which is reflected in high resting levels of free OPDA (1-3 nmoles/g fresh weight in *Arabidopsis*). Therefore, mechanisms of OPDA transport and sequestration are likely to contribute to jasmonate signaling. OPDA sequestered in form of MGDG-O may provide an abundant source of OPDA which can be rapidly released (for example, upon wounding) for signaling purposes. Transport of OPDA sequestered in the chloroplast into peroxisomes is a prerequisite for the formation of the cyclopentanone jasmonates, and release into the cytosol may be required for OPDA signaling. The respective transport mechanisms, neither one of which has been identified, are thus likely to play important roles in jasmonate synthesis and signaling.

Metabolites of JA

As a result of the enantiomeric specificity of the AOC-catalyzed reaction, the Vick and Zimmermann pathway yields specifically (+)-7-iso-JA ((3R, 7S)-JA). Due to steric hindrance of the two side chains at the cyclopentanone ring in *cis* configuration, (+)-7-iso- JA readily isomerizes to the thermodynamically favored (-)-JA ((3R, 7R)-JA; Figure 1) which is the predominant form of JA in plant tissues (molar ratio of of 9:1; Quinkert and others 1982). In addition to the two JA isomers, a bewildering variety of metabolites are formed *in planta* (Figure 3) initiated by enzymatic reduction of the carbonyl function and hydroxylations at C11 and C12. *O*-glycosylated derivatives of 11-OH and 12-OH-JA have been detected in plant extracts as well as methyl, glucosyl, and gentobiosyl esters and amide-linked amino acid conjugates of the C1 carboxyl (reviewed in Parthier 1991; Sembdner and Parthier 1993; Wasternack and Hause 2002; Swiatek and others 2004). Although biological activity has been attributed to the cyclopentanol (cucurbic acid)- and 12-OH-derivatives (tuberonic acid), the glycosylated forms and amino acid derivatives have been viewed as mere conjugates of JA which may be important for hormone homeostasis. Similarly in auxin homeostasis, the balance between hormone biosynthesis, hormone degradation, and hormone (de)conjugation determines the actual level of active auxin (Bartel and others 2001). The proven activity of JA-conjugates in numerous bioassays could thus be explained by endogenous hydrolysis and release of JA. When

supplied to excised barley leaves, however, Kramell and others observed negligible interconversion of JA and its amino acids conjugates, indicating that both may be active in regulating jasmonate responsive gene expression (Kramell and others 1997). Furthermore, recent findings in *Arabidopsis* indicate that conjugation is actually a prerequisite for bioactivity in the regulation of at least a subset of JA responses (Seo and others 2001; Staswick and others 2002).

The *Arabidopsis jar1* mutant has been isolated in a screen for reduced sensitivity to JA implying that JA perception or signaling may be affected. *jar1* is moderately resistant to JA in assays for root growth inhibition and is less responsive to MeJA than the wild type with respect to the induction of vegetative storage proteins (Staswick and others 1992). In contrast to the stronger phenotype of the JA-insensitive *coil* mutant including male sterility, *jar1* is fully fertile, indicating that some but not all of the JA responses are affected in this particular mutant (Staswick and others 2002). *JAR1* was found to encode a JA-conjugating enzyme, catalyzing the Mg-ATP-dependent adenylation (activation) of the JA carboxyl group and the subsequent conjugation to Ie, Leu, Phe, and Val *in vitro* (Staswick and others 2002; Staswick and Tiryaki 2004). JA-Ile is the predominant amino acid conjugate in plants (Kramell and others 1997; Hause and others 2000) and the only one that was negatively affected in *jar1*, indicating that this is the product formed by JAR1 *in vivo* (Staswick and Tiryaki 2004). The JA-insensitive phenotype of *jar1* which could be complemented by exogenous application of JA-Ile indicates that conjugation to Ile is required for the subset of JA responses that is affected in the mutant (Staswick and Tiryaki 2004). In contrast to the reduced amounts of JA-Ile, conjugates of JA with Phe and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) were actually increased in *jar1*, indicating the existence of at least one additional JA conjugating enzyme (JCE). The JA-ACC conjugating enzyme may regulate the availability of JA and ACC, and may thus contribute to the coordination of the JA and ethylene signaling pathways (Staswick and Tiryaki 2004) which was initially observed in wounded tomato leaves (O'Donnell and others 1996).

The volatile methyl ester of JA (MeJA) is highly active in bioassays for JA activity and it has been assumed that exogenously applied or airborne MeJA is de-esterified to JA as the primary intracellular signal transducer in plant tissues (Farmer and Ryan 1990; Farmer and Ryan 1992). The G2/M arrest in tobacco BY-2 cells, for example, is mediated specifically by JA which is formed hydrolytically from exogenously applied MeJA (Swiatek and others 2004). A candidate enzyme for this function (methyljasmonate esterase; MJE) has recently been cloned from tomato, its substrate specificity for MeJA, however, remains to be demonstrated (Stuhlfelder and others 2004). The characterization of a JA carboxyl methyltransferase (JMT), however, indicates that the opposite may also be true: formation of MeJA may actually be necessary for the activation of JA responses (Seo and others 2001). The *JMT* gene has been cloned from *Arabidopsis* and found to encode a highly specific S-adenosyl-L-methionine: JA carboxyl methyltransferase. Transgenic *Arabidopsis* plants constitutively expressing the *JMT* gene had eleveated levels of MeJA while the JA content remained unchanged. JA-responsive genes were expressed constitutively and enhanced resistance against *Botrytis cinerea* was observed in the overexpressors. The data suggest that the formation of the methyl ester activates JA as a signal in the defense response (Seo and others 2001).

Even more volatile than MeJA is *cis*-jasmone (*cis*J), a decarboxylated derivative of JA generated by oxidative degradation of JA via 1,2-didehydro-JA as an intermediate (Koch and others 1997). Unlike JA, 1,2-didehydro-JA and *cis*J were found to be inactive in bioassays (induction of volatile emission) in several plant species. Therefore, the formation of *cis*J was suggested to be an efficient way for the inactivation of JA and the disposal into the gas phase as an infinite sink (Koch and others 1997). In *Nicotiana attenuata*, however, only a small fraction of the herbivore-elicited JA burst was channeled into MeJA and *cis*J, and neither one was recovered in the headspace, suggesting that *Nicotiana* plants do not dispose of JA in the form of its volatile derivatives (von Dahl and Baldwin 2004; Halitschke and Baldwin this issue). Furthermore, Birkett and colleagues reported a function for *cis*J as a semiochemical in plant defense (Birkett and others 2000). *cis*J was shown to be active on three trophic levels by inducing volatile emission in bean plants (1^{st} level), repelling aphids (2^{nd} level), and attracting aphid antagonists (3^{rd} level; Birkett and others 2000; Halitschke and Baldwin this issue).

In the light of the above discussion, the question arises of whether or not there are any inactive JA metabolites at all. It may very well be that there is no general answer to this question: JA may be metabolized for inactivation in one species, while in an other species the very same compound may serve a specialized signaling function. Tuberonic acid glucoside, for example, has been isolated from potato plants and is believed to be the transport

form of tuberonic acid (12-OH-JA) which has tuber-inducing activity in potato (Yoshihara and others 1989; Wasternack and Hause 2002). In most bioassays, however, the compound is inactive: tuberonic acids fails to activate the JA-responsive promoter of cathepsin D inhibitor (Ishikawa and others 1994), it is not a signal in mechanotransduction in *Bryonia* (Blechert and others 1999), it does not cause the accumulation of JA-inducible proteins in barley (Miersch and others 1999), and lacks inhibitory activities on plant growth (Koda 1992). Also in *Arabidopsis*, tuberonic acid fails to induce the expression of the JA-responsive *Thi2.1* gene (Gidda and others 2003). In fact, hydroxylation followed by sulfonation has recently been suggested as a pathway for JA inactivation in *Arabidopsis*, and the sulfotransferase (ST2a) which catalyzes the highly specific sulfonation of 11- and 12-OH-JA (OHJA; Figure 3) has been cloned and characterized (Gidda and others 2003). Alternatively, if tuberonic acid has yet-to-be-identified signaling activity in *Arabidopsis* the sulfotransferase may be required to control its concentration and hormonal activity (Gidda and others 2003).

Conclusions

In recent years, the combination of genetic (mutant analyses) and biochemical (enzyme characterization) approaches has considerably advanced our understanding of individual steps in jasmonate biosynthesis and metabolism. Despite the progress made, certain aspects are still unclear and warrant further investigation. This includes the question of what the substrates are and how they are fed into the jasmonate biosynthetic pathway. Are the initial steps of jasmonate biosynthesis conducted on esterified and/or free fatty acids? With the exception of DAD1, the lipases in jasmonate biosynthesis have not been identified and the mechanisms and signals that trigger lipase activation and the release of jasmonate precursors under different conditions (for example, in response to wounding) are also still elusive. The characterization of the lipase(s) and their substrate specificity likely will resolve the question above. Cyclopentenone and cyclopentanone signaling molecules are synthesized in different subcellular compartments, the plastids and peroxisomes, and release into the cytosol is considered necessary for signaling. The mechanisms and molecular bases for intra- and inter-organellar transport are entirely unknown. The regulation of transport provides an additional level of control in jasmonate signaling and, therefore, the characterization of the machinery involved will be of utmost importance. Recent data indicate that biosynthetic precursors and conjugated derivatives rather than JA itself are active as signal molecules for at least a subset of the jasmonate responses. These findings highlight the importance of (de)conjugating enzymes in the generation of jasmonates with different signaling properties. Further work will be required to uncover the full breadth of specificity afforded by variations of the JA structure. An additional level of complexity is provided by interactions with other hormonal and stress signaling pathways. This topic has not been covered in the present article, but the question of how jasmonate signaling is integrated with other signaling molecules is likely to attract further attention in the future.

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