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Jasmonate biosynthesis in *Arabidopsis thaliana* requires peroxisomal β-oxidation enzymes – Additional proof by properties of *pex6* and *aim1*

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Abstract

Jasmonic acid (JA) is an important regulator of plant development and stress responses. Several enzymes involved in the biosynthesis of JA from α-linolenic acid have been characterized. The final biosynthesis steps are the β-oxidation of 12-oxo-phytoenoic acid. We analyzed JA biosynthesis in the Arabidopsis mutants *pex6*, affected in peroxisome biogenesis, and *aim1*, disrupted in fatty acid β-oxidation. Upon wounding, these mutants exhibit reduced JA levels compared to wild type. *pex6* accumulated the precursor OPDA. Feeding experiments with deuterated OPDA substantiate this accumulation pattern, suggesting the mutants are impaired in the β-oxidation of JA biosynthesis at different steps. Decreased expression of JA-responsive genes, such as *VSP1*, *VSP2*, *AtJRG21* and *LOX2*, following wounding in the mutants compared to the wild type reflects the reduced JA levels of the mutants. By use of these additional mutants in combination with feeding experiments, the necessity of functional peroxisomes for JA-biosynthesis is confirmed. Furthermore an essential function of one of the two multifunctional proteins of fatty acid β-oxidation (AIM1) for wound-induced JA formation is demonstrated for the first time. These data confirm that JA biosynthesis occurs *via* peroxisomal fatty acid β-oxidation machinery.

Keywords: Jasmonate biosynthesis; Arabidopsis; β-Oxidation; *aim1*; *pex6*; Peroxisome

1. Introduction

Jasmonates are ubiquitous plant growth regulators with essential roles in plant development and responses to biotic and abiotic stress. The term jasmonates is used for the free acid (JA), the methyl ester (JAME), and derivatives such as JA-amino acid conjugates (reviewed in Wasternack, 2006). Jasmonates are lipid-derived signals originating from α-linolenic acid (C18:3) in chloroplast membranes (Fig. 1). JA biosynthesis is initiated by insertion of molecular oxygen into position C-13, catalyzed by a 13-lipoxygenase (13-LOX) (Feussner and Wasternack, 2002). The resulting 13-hydroperoxide (13*S*-hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic acid, 13-HPOT) is converted to an unstable allene oxide by an allene oxide synthase (AOS) and cyclized by an allene oxide cyclase (AOC) to *cis*-(+)-12-oxophytodienoic acid (OPDA), which carries the enantiomeric structure of naturally-occurring jasmonates. The corresponding activities of 13-LOX, AOS, and AOC using 16:3 fatty acids as the substrate lead to the formation of dinor-OPDA (Weber et al., 1997). Subsequent reduction to 12-oxophytoenoic acid (OPC-8) is catalyzed by an OPDA reductase (OPR3). Lastly, three rounds of β-oxidation of the carboxylic acid side chain would form

Abbreviations: ACX, acyl CoA oxidase; ANOVA, analysis of variance; AOC, allene oxide cyclase; AOS, allene oxide synthase; CTS, comatose; 2,4-DB, 2,4-dichlorophenoxybutyric acid; 13-HPOT, 13*S*-hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic acid; IBA, indole butyric acid; IAA, indolyl acetic acid; JA, jasmonic acid; JA-ME, JA methyl ester; LOX, lipoxygenase; KAT, 3-ketoacyl-CoA thiolase; MFP, multifunctional protein; OPC-8, 12-oxophytoenoic acid; OPDA, 12-oxophytodienoic acid; OPR, OPDA reductase; VSP, vegetative storage proteins; PP2A, serine/threonine protein phosphatase 2A; PUFA, polyunsaturated fatty acid.

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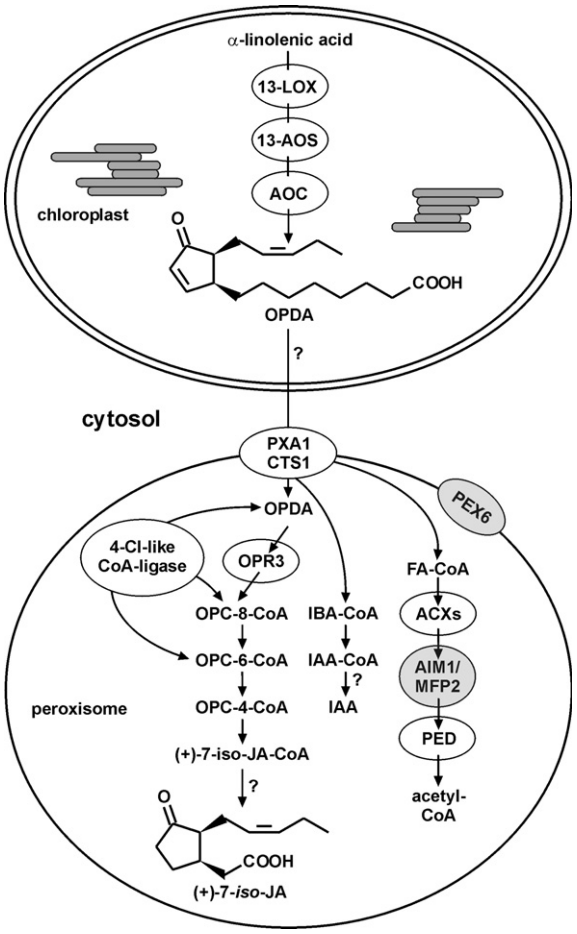

Fig. 1. Metabolic scheme of JA biosynthesis, indicating the subcellular compartments of each step. Additional peroxisomal processes, including IBA and fatty acid β-oxidation, are shown for reference. See text for details.

(+)-7-*iso*-JA, which equilibrates to the more stable (−)-JA. The role of fatty acid β-oxidation in JA biosynthesis initially was suggested by feeding experiments of deuterated OPC derivatives carrying a carboxylic acid side chain with odd or even numbers of carbon atoms to tomato leaves (Miersch and Wasternack, 2000) and by loss of JA-like elicitor properties of compounds structurally altered in the carboxylic acid side chain (Blechert et al., 1995, 1999).

Within JA biosynthesis, the first four genes encoding JA biosynthetic enzymes have been identified in several plant species, including *Arabidopsis thaliana* (Browse, 2005; Delker et al., 2006). In Arabidopsis, six cDNAs encoding LOXs (Feussner and Wasternack, 2002; Bell and Mullet, 1993; Melan et al., 1993), one AOS (Laudert et al., 1996), and four AOCs (Stenzel et al., 2003) have been characterized. Three OPRs have been found, of which apparently only OPR3 is active in JA biosynthesis (Müssig et al., 2000; Schaller et al., 2000). The LOXs, AOS, and AOCs carry functional chloroplast targeting sequences, as confirmed by immunocytological and import studies (Laudert et al., 1996; Froehlich et al., 2001; Stenzel et al., 2003). In contrast, the OPR3 enzyme carries a peroxisomal targeting signal (Stintzi and Browse, 2000) and was localized immunocytochemically in peroxisomes (Strassner et al., 2002).

Peroxisomes are the main compartment of plant cells in which fatty acid β-oxidation occurs (Baker et al., 2006). Under specific conditions, however, such as carbohydrate starvation, mitochondrial β-oxidation may occur (Bode et al., 1999). Although OPR3 localization in peroxisomes suggests that peroxisomal β-oxidation might be involved in JA biosynthesis (converting OPC-8 to JA), a final proof was missing until recently. However, three *Arabidopsis* 4-coumarate:CoA ligase-(4CL)-like proteins with specific activity *in vitro* on OPDA, OPC-8, and OPC-6 were localized in peroxisomes (Schneider et al., 2005; Koo et al., 2006). Identification of these enzymes suggests a mechanism for how OPDA and OPR3 products are activated for β-oxidation. β-oxidation proceeds via an acyl-CoA oxidase (ACX), which catalyzes the first step in fatty acid β-oxidation, followed by a multifunctional protein (MFP) and a L-3-Ketoacyl-CoA thiolase (KAT) (Schilmiller and Howe, 2005). An indication of specific isozyme functions was given recently by reduced JA formation in the double *acx1/5* mutant, which is affected in two members of the *ACX* gene family of *A. thaliana* (Schilmiller et al., 2007). Other proofs for action of fatty acid β-oxidation enzymes in JA biosynthesis came from *ACX1* and *KAT2* mutants and transgenic approaches in *A. thaliana* (Cruz Castillo et al., 2004; Afitlhile et al., 2005; Pinfield-Wells et al., 2005). Even though these single mutants and transgenic lines were clearly affected in JA biosynthesis, they still exhibited considerable amounts of JA, suggesting redundancy among gene family members involved. This possibility strongly emphasizes the need for additional mutants and approaches. Some relevant hints can be drawn from conversion of the endogenous auxin indole butyric acid (IBA) into indole acetic (IAA) (Bartel et al., 2001). Interestingly, mutants affected in fatty acid β-oxidation and peroxisome biogenesis were isolated by resistance to IBA or its analog 2,4-dichlorophenoxybutyric acid (2,4-DB), suggesting that the conversion of IBA into IAA parallels peroxisomal fatty acid β-oxidation (Bartel et al., 2001) (Fig. 1). Three mutants disrupted in fatty acid β-oxidation have been identified in these screens: *acx1* and *acx3* are affected in acyl-CoA oxidase enzymes (Eastmond et al., 2000; Adham et al., 2005) and *ped1* is affected in a thiolase (Hayashi et al., 1998). *aim1* is disrupted in one of two genes encoding multifunctional proteins and is also resistant to 2,4-DB (Richmond and Bleecker, 1999). In addition, the IBA-response mutants *pxa1/ped3/cts1* are affected in the ABC transporter-like protein PXA1, which corresponds to COMATOSE (CTS) and is predicted to import substrates into peroxisomes (Zolman et al., 2001; Footitt et al., 2002; Hayashi et al., 2002; Theodoulou et al., 2005) and the *pex4*, *pex5*, *pex6*, and *ped2/pex14* mutants are affected in peroxisomal biogenesis or matrix protein import (Zolman et al., 2000, 2005; Zolman and Bartel, 2004; Hayashi et al., 2000).

Despite several genetic evidences for function of fatty acid β-oxidation enzymes in JA biosynthesis, the considerable JA levels in analyzed mutants prompted us to study other mutants as well as to perform a feeding experiment and a metabolite analysis covering all intermediates between OPDA and JA. Here we show that the *pex6* and *aim1* mutants accumulate only residual amounts of JA upon wounding. Feeding experiments with deuterated OPDA and analysis of intermediate accumulation revealed that both mutants are affected in β-oxidation of the carboxylic acid side chain. The impaired JA accumulation also is reflected by decreased JA-induced gene expression. The data indicate that AIM1 acts in JA biosynthesis and confirm by additional mutant analysis and feeding experiments that the peroxisomal fatty acid β-oxidation machinery is involved directly in JA biosynthesis.

2. Results

2.1. pex6 and aim1 mutants are impaired in wound-induced formation of JA

To determine if peroxisomal β-oxidation plays a role in JA biosynthesis, we examined JA levels in mutants defective in peroxisomal function (*pex6*) or fatty acid β-oxidation (*aim1*). Wild type rosette leaves grown under normal conditions contain small amounts of JA and higher levels of OPDA intermediates (Fig. 2), as previously described (Laudert and Weiler, 1998; Stintzi et al., 2001). A characteristic feature of many plants, including *Arabidopsis*, is a dramatic increase in JA and OPDA following wounding (Fig. 2) (Stenzel et al., 2003; Laudert and Weiler, 1998; Stintzi et al., 2001). However, unlike wild type, *pex6* and *aim1* showed a significantly less substantial accumulation of JA upon wounding. These results are similar to the JA biosynthetic mutant *opr3* (Fig. 2), which is defective in OPC-8 production from OPDA (Stintzi and Browse, 2000; Stintzi et al., 2001). OPDA levels in *pex6* were elevated compared to wild type, both in unwounded tissue and quite dramatically upon wounding. In addition to JA, the amount of C-d carrying a six and four carbon atom carboxylic side chain (OPC-6 and OPC-4, respectively) was lower in *pex6*. The *aim1* mutant had approximately wild type levels upon wounding of all JA precursors, including OPDA and OPC-4. These data suggest that the two mutants are affected in the conversion of OPDA to JA, possibly at different points.

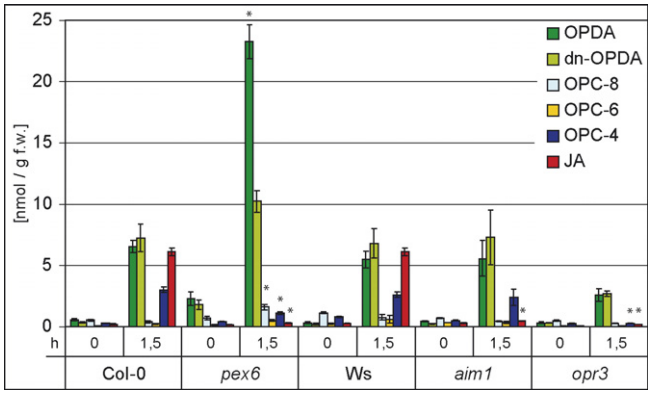

Fig. 2. Wound-induced increase of OPDA, dinor-OPDA, OPC-derivatives, and JA in wild type, *pex6* (Col-0 background), *aim1*, and *opr3* (Ws background). Samples were taken immediately preceding (0 h) and 1.5 h following wounding treatments. Three independent sets of pooled leaf tissue from nine plants each were extracted and subjected to HPLC and GC/MS analysis. Data is shown as nmol product per g f.w. Error bars represent standard errors of the means. Asterisks mark significant differences ($p < 0.05$) between mutants and corresponding wild types as tested by ANOVA and Tukey-HSD-Test.

2.2. pex6 and aim1 are partially impaired in accumulation of C-d derivatives

To substantiate these data, we conducted a feeding experiment using OPDA deuterated in the cyclopentenone ring at carbon atom five. Plants were wounded 0.5 h after application of labeled OPDA. Unlabeled and labeled JA and OPC-derivatives were measured 1.5 h after wounding and compared with plants that were not wounded (Fig. 3) or treated with a mock solution (data not shown).

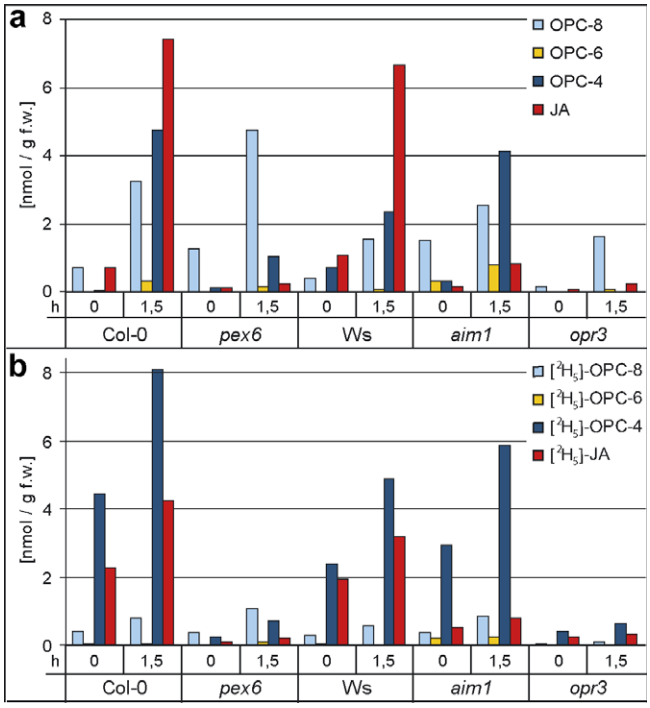

Fig. 3. Wound-induced levels of JA and OPC derivatives in *pex6*, *aim1*, and *opr3* mutant plants examining endogenous accumulation (a) and deuterated compounds (b) following [$^2H_5$]-OPDA feeding. Nine plants each were painted with a 150 μl of a 1 mM [$^2H_5$]-OPDA solution (98% isotopically pure) per ~0.5 g f. w. After 30 min, leaves were harvested (0 h) or wounded and harvested after 1.5 h. Pooled leaf tissues from nine plants each were extracted, purified by HPLC, and subjected to GC/MS analysis to determine non-labeled and deuterated compounds in parallel. Two independent experiments were performed with a measuring range of 15%. One representative experiment is shown.

Accumulation of [$^2H_5$]-C-d and [$^2H_5$]-JA reflect conversion of the exogenous [$^2H_5$]-OPDA. Within 30 min of application (0 h in Fig. 3b), substantial [$^2H_5$]-OPDA was converted into [$^2H_5$]-OPC-4 and [$^2H_5$]-JA in wild type leaves, indicating the effect of substrate feeding in JA biosynthesis. In *pex6*, however, only residual amounts of deuterated compounds were found, illustrating the low capacity of *pex6* to β-oxidize OPC-derivatives. In *aim1* leaves, [$^2H_5$]-OPC-4 accumulated but [$^2H_5$]-JA levels remained low. This accumulation pattern of deuterated compounds was amplified following wounding: (i) both wild types accumulated higher levels of [$^2H_5$]-JA and [$^2H_5$]-OPC-4, (ii) the mutants showed only residual levels of [$^2H_5$]-JA, (iii) *aim1* accumulated [$^2H_5$]-OPC-4, and (iv) in *pex6*, most of the deuterated compounds remained at low level.

[$^2H_5$]-OPDA feeding led to changes in endogenous unlabeled JA and C-d from that shown in Fig. 2. Upon wounding, Col-0 and to lesser extent Ws accumulated OPC-8, whereas other C-d and JA were at similar levels as in non-fed wounded leaves (Fig. 3a versus Fig. 2). The mutants, however, again accumulated only residual JA. The *pex6* mutant accumulated higher unlabeled OPC-8 levels, indicating less conversion and/or activation of this compound. In wounded *opr3*, some OPC-8 was found (Fig. 3a) even though the mutant is impaired in OPC-8 formation (Stintzi and Browse, 2000).

2.3. JA-induced alteration of gene expression is decreased in *aim1* and *pex6*

Several genes are specifically expressed in response to JA, including the *VSP* genes encoding VEGETATVE STORAGE PROTEIN 1 and 2 (Stintzi et al., 2001), *LOX2* (Bell and Mullet, 1993) and *AtJRG21* (Nickstadt et al., 2004). We examined whether the decreased JA levels

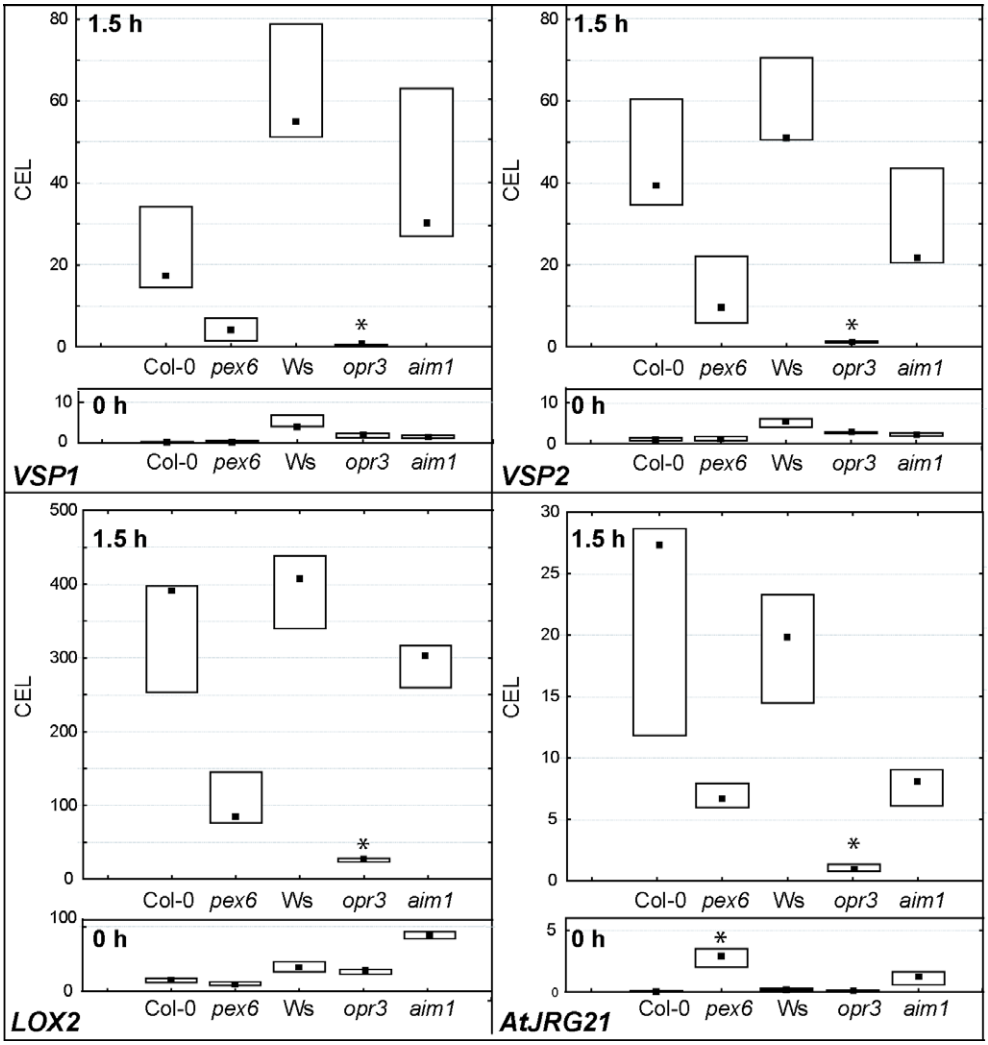

Fig. 4. Relative transcript levels of *VSP1*, *VSP2*, *LOX2* and *AtJRG21*. Black squares represent median values of comparative expression levels (CEL) of three independent biological replicates each taken from pooled leaf material of three to four five-week-old plants. White rectangles show 25–75% percentiles which in this case also represent the min–max range. Asterisks mark significant differences ($p < 0.05$) between mutant and the corresponding wild type as tested by Kruskal-Wallis-ANOVA (*H*-test) and Nemenyi-test.

in wounded *pex6* and *aim1* (Figs. 2 and 3) are reflected by a decrease in mRNA accumulation of these JA-responsive genes compared to wild type. As shown in Fig. 4, *VSP1*, *VSP2*, *LOX2* and *AtJRG21* expression in wild type, *aim1*, and *pex6* increases following wounding. However, mutant leaves accumulate considerably less mRNA of these genes following wounding. In the JA-deficient *opr3* unaltered expression levels were detected following wounding (Fig. 4), as previously indicated by microarray data (Stintzi et al., 2001; Taki et al., 2005).

3. Discussion

The formation of acetyl-CoA by fatty acid β-oxidation, the catabolism of aliphatic amino acids, and the metabolism of glycolate during photorespiration occur in plant peroxisomes (Bartel et al., 2001; Baker et al., 2006). Additionally, the final steps in the conversion of the auxin IBA into IAA is thought to occur in peroxisomes (Bartel et al., 2001). Here, we further examine the role of fatty acid β-oxidation enzymes in the peroxisome acting in the ultimate reactions of JA biosynthesis.

The initial steps of JA biosynthesis take place in chloroplasts, whereas the intermediate OPDA is reduced by the peroxisomal OPR3 (Stintzi and Browse, 2000; Strassner et al., 2002). OPR3 and β-oxidation proteins are targeted to peroxisomes (Reumann et al., 2004). Although nothing is known regarding the export of OPDA or an OPDA-like compound from the chloroplast, recent reports indicate that OPDA is transported into peroxisomes *via* the ABC transporter PXA1/CTS/PED3 acting in fatty acid and IBA import (Theodoulou et al., 2005) and is activated by a peroxisomal OPDA:CoA ligase (Schneider et al., 2005). These results suggest that reduction of OPDA by OPR3 may take place on the OPDA-CoA ester. Activation by the 4CL-like acyl-CoA ligases of the OPR3 product OPC-8 and of OPC-6 have been also shown (Schneider et al., 2005; Koo et al., 2006) which suggests two parallel pathways of activation/conversion in JA biosynthesis. The final steps in JA biosynthesis would require the consecutive action of acyl-CoA oxidases (ACXs), a multifunctional protein (AIM1/MFP2) with enoyl-CoA hydrolase and β-hydroxyacyl-CoA dehydrogenase activity, and a 3-ketoacyl-CoA thiolase (KAT) (Graham and Eastmond, 2002). A role for ACX1 and KAT2 in wound-induced JA biosynthesis of Arabidopsis was suggested based on increased gene expression upon wounding and JA application as well as by a reduction in wound-induced JA formation in corresponding antisense-plants (Cruz Castillo et al., 2004). Furthermore, cloning of the tomato *ACX1A* revealed that the encoded protein is essential for wound- and herbivore-induced JA formation (Li et al., 2005) and the *acx1/5* double mutant was shown to be affected in JA biosynthesis (Schilmiller et al., 2007).

Using further mutants combined with a feeding experiment of the deuterated JA-precursor OPDA in Arabidopsis, we show that the peroxisomal fatty acid β-oxidation machinery is responsible for β-oxidation of the carboxylic acid side chain of OPC-8 in JA biosynthesis. Three types of mutants were used: (i) *pex6*, which is affected in a gene encoding a protein necessary for protein import during peroxisome biogenesis (Zolman and Bartel, 2004), (ii) *aim1*, which is affected in one of the two Arabidopsis MFPs (Richmond and Bleecker, 1999) and (iii) *opr3*, which is affected in a gene encoding the OPDA reductase OPR3 (Stintzi and Browse, 2000) and served as a control.

Interestingly, *pex6* and *aim1* seedlings are dependent on sucrose during early seedling development and are resistant to IBA and 2,4-DB treatments, indicating defects in β-oxidation of fatty acids and in the conversion of IBA to IAA (Richmond and Bleecker, 1999; Zolman et al., 2000; Zolman and Bartel, 2004). In both mutants, JA deficiency was significant following wounding (Figs. 2 and 3), similar to the well-characterized JA-deficient *opr3* mutant (Stintzi et al., 2001) and was accompanied with a reduced induction of JA-responsive genes (Fig. 4).

JA deficiency of the mutants was observed in the endogenous content as well as upon feeding of deuterated OPDA (Figs. 2 and 3). Within this feeding experiment there is no dilution of deuterated OPDA and downstream intermediates of JA biosynthesis (Fig. 3) which corresponds to similar data detected for tomato plants (Miersch and Wasternack, 2000). Therefore, the remarkable amount of esterified OPDA and esterified intermediates known to be released upon wounding of *A. thaliana* leaves (Stelmach et al., 2001; Hisamatsu et al., 2003) was out of influence on this labeling experiment. The wound-induced accumulation of OPDA and dinor-OPDA known for both wild types was also observed in *pex6*, *aim1*, and *opr3* (Fig. 2), indicating that the mutants are impaired downstream of OPDA formation. The increased accumulation of OPDA, dinor-OPDA, and OPC-8 in *pex6* compared to wild type may be due to general peroxisomal defects in *pex6*, putatively including the import of OPR3, which acts on OPDA, and other β-oxidation proteins. Alternatively, *aim1* has a the single enzymatic defect and shows a distinct accumulation pattern (Fig. 3a). A second multifunctional protein, MFP2, may have overlapping activity in the first two β-oxidation reactions, whereas AIM1 could be singularly responsible for OPC-4 CoA metabolism, resulting in the wild-type levels of OPC-4, but reduced accumulation of JA. The accumulation of OPC-8 in *opr3* in feeding experiments, even though this mutant is impaired in OPC-8 formation, suggests that OPR1 and OPR2, which are not specific to the four enantiomeric forms of OPDA, may be able to convert *cis*-(+)-OPDA under conditions of strongly increased substrate availability as occurring by exogenous feeding. In contrast, without exogenous feeding, no OPC-8 accumulates, indicating a high substrate specificity of OPR3 (Fig. 2). The minor but specific differences in the substrate binding pocket of OPR1 and OPR3 shown upon crystallization of both proteins suggests that such differences

between exogenous and endogenous OPDA may occur (Breithaupt et al., 2001, 2006).

A characteristic feature of the labeling experiment with deuterated OPDA (Fig. 3) is the common accumulation of OPC-4 and JA in both unwounded and wounded wild-type samples. This result is indicative of possible chain-length specificity in JA precursor β-oxidation. Interestingly, the capacity of tomato leaves to form JA upon OPC-8, OPC-6, or OPC-4 treatment gradually decreased, suggesting that the efficiency of the compounds to enter JA-forming reactions decreases with shorter side chains (Miersch and Wasternack, 2000). In addition, three independent 4-CL-like enzyme activities activating OPDA, OPC-8 or OPC-6 were found, supporting chain-length specificity at the acyl-CoA ligase step (Schneider et al., 2005; Koo et al., 2006). Furthermore, the ACX enzymes also exhibit chain length specificity: among the five Arabidopsis *ACX* genes, *ACX3* encodes a medium-chain length acyl-CoA oxidase (Eastmond et al., 2000; Froman et al., 2000), whereas *ACX4* encodes a short-chain length acyl-CoA oxidase (Rylott et al., 2003). Perhaps the OPC-8, OPC-6, and OPC-4 substrates are differentially catalyzed by ACX gene family members as supported by distinct decreases in wound-induced JA accumulation in *acx1*, *acx5*, and *acx1/5* mutants (Schilmiller et al., 2007).

There are strong arguments for regulation of JA biosynthesis by a positive feed back loop and by substrate availability. Arabidopsis *AOS* overexpression lines (Laudert et al., 2000; Wang et al., 1999) and wild-type leaves that accumulate LOX, AOS, and AOC (Stenzel et al., 2003) generate JA only upon external stimuli, such as wounding. The substrate deficiency in JA biosynthesis also is documented in the [$^2H_5$]-OPDA labeling experiment (Fig. 3). Feeding elevates levels of [$^2H_5$]-OPC derivatives and of [$^2H_5$]-JA in unwounded leaves and is further increased upon wounding. Among the OPC-derivatives with different length side chains, only compounds with an even number of carbon atoms accumulate, as expected for shortening by β-oxidation. This corresponds to previously found defects in JA formation and a lack of proteinase inhibitor2 (*PIN2*) mRNA accumulation in tomato leaves upon feeding of OPC-derivatives with a carboxylic acid side chain of three, five, or seven carbon atoms (Miersch and Wasternack, 2000).

The impaired wound-induced formation of JA in *pex6* and *aim1* also is reflected in the considerably reduced induction of the JA-responsive genes such as *VSP1*, *VSP2*, *LOX2* and *AtJRG21* (Fig. 4). The expression of *VSP* genes and *LOX2* is commonly used as a specific marker for JA-formation (Stintzi et al., 2001; Jensen et al., 2002; Taki et al., 2005; Jung et al., 2007). The promoters of these genes have been shown to be JA-responsive (Ellis and Turner, 2001; Jensen et al., 2002) and *AtJRG21* also is JA-inducible (Nickstadt et al., 2004). Due to the high sequence similarity of the two *VSP*-genes, a specific detection of the individual *VSP*-transcripts by Northern blot or ATH1-microarray analysis is hindered. Most of the published data based on these analyses give an impression of *VSP*-expression in total rather than expression levels of specific *VSP*s. Here we used a quantitative real-time RT-PCR-method for the specific detection of *VSP1*- and *VSP2*-transcript levels thereby providing evidence that both *VSP*-transcripts accumulate in response to wounding (Fig. 4). Considerable differences were found between the wild type and the *aim1* and *pex6* mutant, respectively, whereas the JA-deficient *opr3* mutant lacks induction of JA-responsive genes upon wounding.The higher wound-induced accumulation of all transcript levels in *pex6* and *aim1* in comparison to *opr3* reflects the higher observed capacity of wound-induced JA-formation in *pex6* and *aim1* (Figs. 2 and 3). This phenomenon was also found with mutants affected in *ACXs* or genes encoding 4-CL-like ligases, due to redundancy within these gene families (Koo et al., 2006; Schilmiller et al., 2007). Furthermore, OPC-8, OPC-6 and OPC-4 are known to induce JA-responsive genes such as that coding for the *PIN2* of tomato (Miersch and Wasternack, 2000). Consequently, diminished expression of JA responsive genes due to lower JA levels in *aim1* and *pex6* could be compromised by OPC-derivatives.

With this work, we clearly demonstrated the diminished capacity of *pex6* and *aim1* for wound-induced formation of JA, thereby confirming the requirement of functional peroxisomes and the involvement of fatty acid β-oxidation enzymes in JA-biosynthesis.

4. Experimental

4.1. Chemicals

(±)-JA was prepared by saponification of racemic JAME (Firmenich Geneva). [$^2H_5$]-OPC-4 (3-*oxo*-2-(2*Z*-pentenyl)-cyclopentane-1-butyric acid) was electrochemically synthesized *via* Kolbe synthesis (Hamberg, 1988). [$^2H_5$]-OPC-8 (3-*oxo*-2-(2*Z*-pentenyl)-cyclopentane-1-octyric acid) was prepared as described (Miersch and Wasternack, 2000).

The deuterated standards [$^2H_6$]-JA and [$^2H_5$]-OPDA were prepared according to Miersch et al. (1991) and Zimmermann and Feng (1978), respectively.

4.2. Plant material, growth and incubation conditions

opr3 seeds (Wassilewskija, Ws background) were kindly provided by Prof. J. Browse (Washington, USA) (Stintzi and Browse, 2000) and Dr. A. Stintzi (Stuttgart-Hohenheim, Germany). *aim1* seeds (Ws background) were kindly provided by Dr. T. Richmond (Wisconsin, USA) (Richmond and Bleecker, 1999). *pex6* seeds (Columbia, Col-0 background) have been described previously (Zolman et al., 2000; Zolman and Bartel, 2004). Plants were cultivated in controlled chambers (Percival, CLF) at 70% relative humidity under 16 h light and 210 $\mu E\, m^{-2}\, s^{-1}$ for 3 weeks. Wounding was performed by crushing leaves once

with forceps across the mid-vein. Feeding experiments with deuterated compounds were performed on three week old plants by painting 150 µl per 0.5 g f. w. of [$^2H_5$]-OPDA (1 mM in 1% acetone and 0.5% Tween20). Control plants were treated with equal amounts of dH_2O in 1% acetone and 0.5% Tween20. Because wound-induced accumulation of jasmonates in *A. thaliana* leaves peaks between 1 and 2 h (Laudert and Weiler, 1998; Stintzi et al., 2001; Stenzel et al., 2003) we performed sampling at one representative time point (1.5 h) after wounding.

4.3. Extraction, purification and quantification of JA, OPDA, dinor-OPDA and OPC derivatives

Leaf tissue of nine plants (0.3–0.5 g f. w.) was frozen in liquid nitrogen and homogenized in a mortar. Extraction, separation by HPLC, and quantitative analysis by GC/MS were performed as described (Miersch and Wasternack, 2000; Stenzel et al., 2003).

4.4. RNA extraction and quantitative real time RT-PCR

Five week old plants were harvested unwounded and 1.5 h after wounding, respectively. For each genotype and time-point plant material of three to four plants was pooled and homogenized in liquid nitrogen. Total RNA was extracted from 50 to 100 mg leaf tissue by Qiagen RNeasy Mini Kit including an on-column DNase digestion. After quality control by native gel electrophoresis 3 µg of total RNA were used for first strand cDNA synthesis by Superscript III reverse transcriptase (Invitrogen) following the manufacturers instructions. Quantitative real time PCR was performed in a Mx3005P™ QPCR System (Stratagene) using the Power SYBR® Green PCR Master Mix (Applied Biosystems). For each reaction 20 ng of total cDNA were used as template for generation of *VSP1* (*At5g24780*), *VSP2* (*At5g24770*), *AtJRG21* (*At3g55970*) and *LOX2* (*At3g45140*) amplicons using primers *VSP1-582F* 5′-CACTGTCGAGAATCTCAAGGCTG-3′, *VSP1-646R* 5′-CGTTTGGCTTGAGTATGAGATGC-3′, *VSP2-553F* 5′-TTGTGGAAGAATGTCACTCTCGAC-3′ and *VSP2-626R* 5′-GGCTTCAATATGAGATGCTTCCAG-3′, *LOX2-F* 5′-TGCACGCCAAAGTCTTGTCA-3′, *LOX2-R* 5′-TCAGCCAACCCCCTTTTGA-3′, *AtJRG21-F* 5′-TTGACCATTCTCTTGCCGGA-3′ and *AtJRG21-R* 5′-GCATCTGAATTTGGTCACCCA-3′, respectively. The cDNA of a *PP2A* (*At1g13320*) was amplified by the use of primers *PP2A-824F* 5′-AGCCAACTAGGACGGATCTGGT-3′ and *PP2A-896R* 5′-GCTATCCGAACTTCTGCCTCATTA-3′ and served as a constitutively expressed control as described by Czechowski et al. (2005). Comparative expression levels (CEL) of mean Ct-values of two technical replicates for each sample were calculated as $2^{(Ct\ PP2A\text{-}Ct\ gene\ of\ interest)}$. CEL of three independent biological replicates were subjected to Kruskal-Wallis ANOVA and Nemenyi-Test in order to test for significant differences between expression levels of mutants and wild types. Data sets of unwounded and wounded samples were tested separately.

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