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Characterization and cDNA-microarray expression analysis of 12-oxophytodienoate reductases reveals differential roles for octadecanoid biosynthesis in the local versus the systemic wound response

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Summary

12-Oxophytodienoate reductases (OPRs) belong to a family of flavin-dependent oxidoreductases. With two new tomato isoforms reported here, three OPRs have now been characterized in both tomato and *Arabidopsis*. Only one of these isoforms (OPR3) participates directly in the octadecanoid pathway for jasmonic acid biosynthesis, as only OPR3 reduces the 9*S*,13*S*-stereoisomer of 12-oxophytodienoic acid, the biological precursor of jasmonic acid. The subcellular localization of OPRs was analyzed in tomato and *Arabidopsis*. The OPR3 protein and activity were consistently found in peroxisomes where they co-localize with the enzymes of β-oxidation which catalyze the final steps in the formation of jasmonic acid. The octadecanoid pathway is thus confined to plastids and peroxisomes and, in contrast to previous assumptions, does not involve the cytosolic compartment. The expression of tomato (*Lycopersicon esculentum, Le*) OPR3 was analyzed in the context of defense-related genes using a microarray comprising 233 cDNA probes. *Le*OPR3 was found to be up-regulated after wounding with induction kinetics resembling those of other octadecanoid pathway enzymes. In contrast to the induction of genes for wound response proteins (e.g. proteinase inhibitors), the accumulation of octadecanoid pathway transcripts was found to be more rapid and transient in wounded leaves, but hardly detectable in unwounded, systemic leaves. Consistent with the expression data, OPDA and JA were found to accumulate locally but not systemically in the leaves of wounded tomato plants. The transcriptional activation of the octadecanoid pathway and the accumulation of JA to high levels are, thus not required for the activation of defense gene expression in systemic tissues.

Keywords: systemin, oxylipins, jasmonic acid, defense signaling, peroxisome, *Lycopersicon esculentum*.

Introduction

Jasmonates, i.e. jasmonic acid (JA) and related cyclopentanones are ubiquitously occurring plant growth regulators. They were originally described to affect a variety of developmental processes including root growth, senescence, fruit ripening, pollen development, and tuber formation (Parthier, 1991). More recently, they have been recognized as signal molecules in plant defense reactions. Jasmonic acid and its methyl ester, as well as its biosynthetic precursors were shown to induce the accumulation of proteinase inhibitors that are part of the inducible herbivore defense system in tomato plants (Farmer and Ryan, 1990, 1992). Following the pioneering work of Farmer and Ryan, a role for jasmonates in the regulation of plant defense reactions against herbivores and pathogens has been firmly established (for review see Blée, 1998; Reymond and Farmer, 1998; Wasternack and Parthier, 1997; Weiler *et al.*, 1998).

Most notably, mutants in *Arabidopsis* and tomato that fail to either synthesize or perceive JA were found to be highly susceptible to insect predators (Howe and Ryan, 1999; Howe *et al.*, 1996; McConn *et al.*, 1997; Xie *et al.*, 1998).

It has been a matter of debate, however, whether jasmonates or rather their cyclopentenone precursors, such as 12-oxophytodienoic acid (OPDA), 15,16-dihydro-OPDA, and dinor-OPDA are the endogenous signaling molecules. These 18- and 16-carbon cyclopentenoic acids are derived from linolenic (18 : 3), linoleic (18 : 2), and hexadecatrienoic (16 : 3) acids, respectively, which are supposed to be released from membrane lipids by specific lipases. Phospholipases (PL) A1, A2, and D have been implicated in this process (Dhondt *et al.*, 2000; Ishiguro *et al.*, 2001; Narváez-Vásquez *et al.*, 1999; Wang *et al.*, 2000). The cyclopentenones are formed via the successive activities of lipoxygenase (LOX), alleneoxide synthase (AOS), and alleneoxide cyclase (AOC). The cyclopentenones are subsequently reduced by OPDA reductase (OPR) yielding, in case of OPDA, 3-oxo-2(2′-pentenyl)-cyclopentane-1-octanoic acid (OPC-8:0), the first cyclopentanone precursor of JA. Three cycles of β-oxidation are then required for the shortening of the OPC-8:0 side chain to yield OPC-2:0, i.e. JA (León and Sánchez-Serrano, 1999; Mueller, 1997; Schaller, 2001; Vick and Zimmerman, 1984).

Evidence for OPDA as a signal molecule in its own right was first derived from the observation that several JA responses are induced by OPDA and its structural mimic coronatine as well (Kutchan, 1993; Weiler *et al.*, 1994). Furthermore, β-oxidation was shown not to be required for phytoalexin induction in *Eschscholtzia* cell cultures (Blechert *et al.*, 1995), indicating that OPDA is active *per se* in this system. Likewise, OPDA appears to be the endogenous signal for mechanotransduction in tendril coiling of *Bryonia dioica*. OPDA and structural analogues that cannot be metabolized to JA were shown to be highly active as mechanotransducers. Furthermore, changes in the levels of endogenous OPDA rather than JA correlated with the progress of the tendril coiling response (Blechert *et al.*, 1999; Stelmach *et al.*, 1998; Weiler *et al.*, 1993). In wounded tomato plants, the accumulation of OPDA precedes that of JA, and in this system, JA was suggested to be an OPDA-metabolite which retains some of the signal molecule's activity (Parchmann *et al.*, 1997). However, this conclusion is in contrast to findings of Miersch and Wasternack (2000), who demonstrated a direct correlation between the amount of JA formed from side chain-modified JA derivatives and the degree of defense gene activation.

The discovery of additional biologically active oxylipins (reviewed by Blée, 1998) led to the concept of the oxylipin signature, predicting a dynamic mixture of JA, OPDA and other bioactive oxylipins to be more relevant for the outcome of a physiological response than the concentration of any one individual signaling molecule (Kramell *et al.*, 2000; Vollenweider *et al.*, 2000; Weber *et al.*, 1997). The concept is supported by the finding that JA and OPDA induce the emission of different blends of volatiles in lima bean implying that any observed blend of volatiles is the combination of individual blends each specifically induced by JA, OPDA, and maybe other signaling molecules (Koch *et al.*, 1999).

The assessment of the relative contributions of JA and OPDA to the induction of various physiological responses was made possible by the isolation of an *Arabidopsis* mutant lacking OPR3 activity, the enzyme catalyzing the committed step in the conversion of OPDA to JA. The mutant was designated *delayed dehiscence 1* (*dde1*, Sanders *et al.*, 2000) as well as *opr3* (Stintzi and Browse, 2000) and, for simplicity, will here be referred to as *opr3*. The mutant is male sterile because of shortened anther filaments, delayed dehiscence of the anther locule, and reduced pollen viability. All these defects 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 *et al.*, 2000; Stintzi and Browse, 2000). Surprisingly, *opr3* was found to be fully resistant to larvae of *Bradysia impatiens* and the fungus *Alternaria brassicicola* demonstrating that OPDA can substitute for JA in the induction of disease and herbivore resistance (Stintzi *et al.*, 2001). While OPDA induced the expression of many JA-responsive genes, a subset of genes was induced in *opr3* by OPDA but not by JA (Stintzi *et al.*, 2001) indicating overlapping as well as distinct functions.

Hence, the characterization of the *Arabidopsis opr3* mutant corroborated the available physiological data showing unequivocally that cyclopentenones (OPDA) and cyclopentanones (JA) have different signaling functions. The reduction of cyclopentenones catalyzed by OPR is, thus likely to be a highly regulated process. Supporting this notion, which stimulated our interest in this enzyme, OPDA was found to accumulate without JA being formed in mechanically stimulated tendrils and in elicited cell cultures (Blechert *et al.*, 1999; Parchmann *et al.*, 1997). Interestingly, the enzymes of cyclopentenone (LOX, AOS, AOC) and cyclopentanone (β-oxidation) biosynthesis are confined to plastids and peroxisomes, respectively (for review see Mueller, 1997; Schaller, 2001). OPR, on the other hand, was hitherto thought to be localized in the cytosol, and thus, three subcellular compartments were thought to participate in the biosynthesis of JA. OPR was first purified as a soluble, presumably cytosolic enzyme from cell cultures of *Corydalis sempervirens* (Schaller and Weiler, 1997a) and sequence information derived from this enzyme allowed the cloning of a first OPR cDNA (*At*OPR1) from *Arabidopsis* (Schaller and Weiler, 1997b). As it turned out, however, *At*OPR1 does not reduce 9*S*,13*S*-OPDA, the only one out of four possible OPDA stereoisomers (cf. Figure 1), that is a precursor of biologically active JA, and the same holds true for the *Arabidopsis OPR2* gene product (Biesgen and

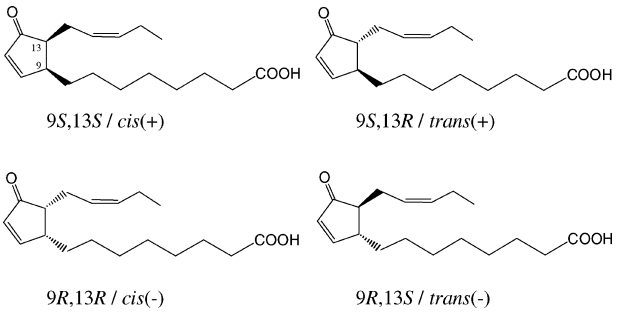

Figure 1. Structures of the four 12-oxophytodienoic acid stereoisomers. 9*S*,13*S*-OPDA (*cis*(+)-OPDA) is the precursor of biologically active jasmonic acid (3*R*,7*S*-JA). Enolization of 9*S*,13*S*- and 9*R*,13*R*-OPDAs yields the respective *trans* isomers. The terminology is the same as the one used for OPC-8:0 isomers which result from OPDA via reduction of the 10,11-double bond.

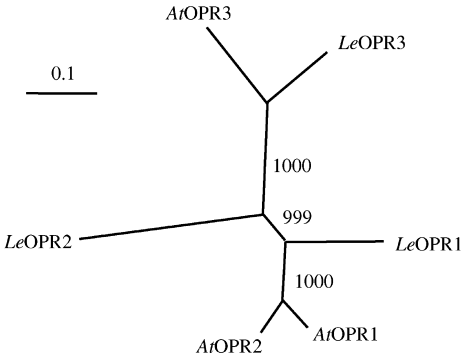

Figure 2. Phylogenetic relationship of tomato and *Arabidopsis* OPR isoforms. The amino acid sequences deduced from OPR cDNAs of tomato and *Arabidopsis* were compared using the program CLUSTALX. The program TREEVIEW was used to generate the phylogenetic tree from the resulting alignment. Bootstrap support of the branches is indicated in the figure (1000 bootstrap runs).

Weiler, 1999; Schaller *et al.*, 2000b; Schaller *et al.*, 1998). Finally, a third isoform (*At*OPR3) was identified in a screen for brassinosteroid-up-regulated cDNAs and this enzyme was the only *Arabidopsis* isoform accepting 9*S*,13*S*-OPDA as its substrate (Müssig *et al.*, 2000; Schaller *et al.*, 2000b). We show here that OPRs 1 and 2 in *Arabidopsis* are cytosolic enzymes whereas *At*OPR3, in contrast to previous assumptions, is localized in peroxisomes. Thus, the JA biosynthetic pathway is confined to plastids and peroxisomes and does not involve the cytosolic compartment.

One OPR isoform (*Le*OPR1) has been cloned and characterized from tomato (Straßner *et al.*, 1999; Zheng *et al.*, 2001). While its stereospecificity has not yet been analyzed biochemically, the available structural and biochemical data indicate some functional similarity with *At*OPR1 (Breithaupt *et al.*, 2001; Straßner *et al.*, 1999).

Here we report the cloning of two new tomato homologues of OPR (*Le*OPR2, *Le*OPR3), the heterologous expression of all three tomato isoforms, the characterization of their stereospecificity, the subcellular localization, and expression in response to wounding. The data indicate a limited role for JA biosynthesis in the activation of wound response gene expression in systemic as compared to wounded tissues. This conclusion is corroborated by the analysis of OPDA and JA concentrations, which increase locally but not systemically after wounding.

Results

Cloning of tomato OPRs

In *Arabidopsis*, three OPR isozymes (*At*OPR1–3) which differ in their substrate specificity for OPDA stereoisomers (cf. Figure 1) have been characterized and a single OPR (*Le*OPR1) has been identified in tomato (Biesgen and Weiler, 1999; Müssig *et al.*, 2000; Schaller and Weiler, 1997b; Schaller *et al.*, 2000b; Straßner *et al.*, 1999). Database searches identified tomato ESTs derived from two transcripts related to *Le*OPR1. The corresponding full-length cDNAs were cloned by rapid amplification of cDNA eucls-PCR (RACE-PCR) and designated *Le*OPR2 and *Le*OPR3, respectively. The *Le*OPR2 cDNA contains an open reading frame of 1065 base pairs coding for a protein with a calculated M_r of 39 426. The first ATG in position 153 is preceded by an in-frame stop codon indicating that the cDNA contains the entire protein-coding region. Likewise, an in-frame stop codon is found upstream of the first ATG (position 93) in the *Le*OPR3 cDNA suggesting that the cDNA is full-length, encoding a protein of 43 525 kDa.

As revealed by pairwise comparisons of the cDNA-deduced amino acid sequences, *Le*OPR1 and 3 are most similar to *At*OPR1 and 3, sharing 72 and 74% of sequence identity, respectively. *Le*OPR2, on the other hand, is only 50, 49, and 42% identical to OPRs 1–3 from *Arabidopsis*. These relationships are confirmed by the phylogenetic analysis shown in Figure 2 suggesting *Le*OPR3 to be the orthologue of *At*OPR3, and *Le*OPR1 to be the nearest neighbor of *At*OPR1 and 2. While *Le*OPR2 is clearly homologous, the common ancestor with this enzyme dates further back in phylogenetic development.

Substrate specificity of tomato OPRs

The three tomato OPRs, *Le*OPR1–3, were expressed in *E. coli* as N-terminal GST-, or $(His)_6$-tagged fusion proteins and were purified from bacterial extracts by affinity chromatography. The apparent molecular mass observed for the predominant band in all three protein preparations during SDS–PAGE corresponded to the mass expected for each of the fusion proteins (Figure 3). The preparation of the GST–*Le*OPR1 fusion protein was contaminated by two rather abundant degradation products. Only the full-length fusion protein, however, exhibited OPR activity

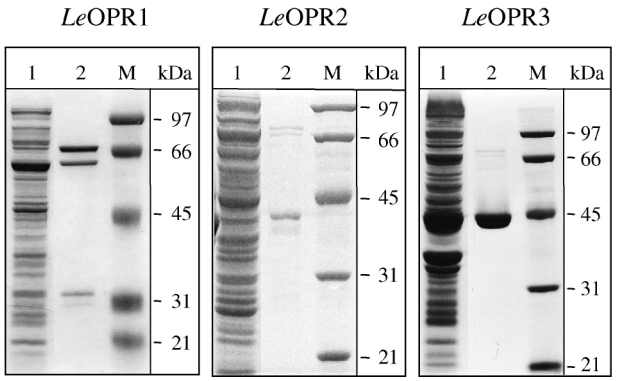

Figure 3. Purification of recombinant *Le*OPRs. *Le*OPR1 (left), *Le*OPR2 (center), and *Le*OPR3 (right) were expressed as N-terminal GST (*Le*OPR1) and $(His)_6$ (*Le*OPR2, 3) tagged fusion proteins in *E. coli*. The recombinant proteins were purified from crude extracts (lanes 1) by affinity chromatography (lanes 2). The molecular weights (in kDa) of marker proteins (Bio-Rad, lanes M) are indicated.

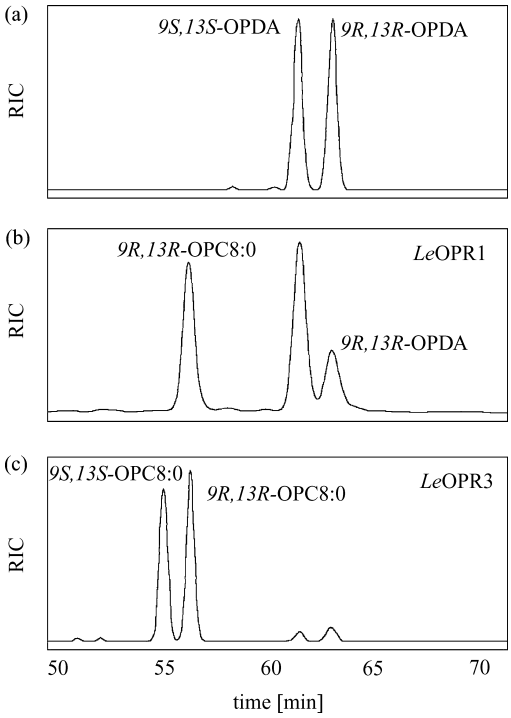

Figure 4. Stereospecificity of tomato OPRs (substrates and products were identified by electron impact (70 eV) mass spectral analyses; data not shown).
(a) The stereospecificity of *Le*OPR1 and 3 was analyzed by chiral capillary GC–MS on a permethyl-α-cyclodextrin stationary phase. The GC traces show the reconstructed ion current (RIC) obtained for the substrate, i.e. 1 mM racemic *cis*-OPDA.
(b) The two peaks are derived from 9*S*,13*S* and 9*R*,13*R*-OPDA as indicated. Upon incubation with *Le*OPR1 (0.75 μg, 30 min, 25°C) 9*R*,13*R*-OPDA is converted to 9*R*,13*R*-OPC-8:0.
(c) Incubation with *Le*OPR3, on the other hand, results in the formation of both 9*R*,13*R*- and 9*S*,13*S*-OPC-8:0 from 9*R*,13*R*- and 9*S*,13*S*-OPDA, respectively.

(Straßner *et al.*, 1999). The preparations of *Le*OPR1 and 3 were strikingly yellow in color and UV–VIS absorption spectra indicated the presence of a tightly bound flavin cofactor (Straßner *et al.*, 1999; data not shown). However, no flavin could be detected in association with *Le*OPR2.

*Le*OPR1 has been characterized previously and was found to catalyze the NADPH-dependent reduction of a broad range of α,β-unsaturated carbonyl compounds including a racemic mixture of OPDA (Straßner *et al.*, 1999). Here we analyzed the stereospecificity of tomato OPRs using chiral capillary gas chromatograpy to separate, and mass spectrometry to identify the different enantiomers in the racemic mixture of substrates and products (Figure 4, Schaller *et al.*, 1998). When the *cis* enantiomers of OPDA (9*S*,13*S*-OPDA, 9*R*,13*R*-OPDA) were used as substrates for *Le*OPR1, only 9*R*,13*R*-OPDA was reduced yielding 9*R*,13*R*-OPC-8:0 (Figure 4b). *Le*OPR3, on the other hand, reduced both enantiomeric forms of *cis*-OPDA giving rise to 9*S*,13*S*-OPC-8:0 and 9*R*,13*R*-OPC-8:0, the former being the precursor of the biologically active jasmonic acid isomer, i.e. 3*R*,7*S*-JA (Figure 4c). In contrast, *Le*OPR2 did not accept OPDA as a substrate nor did it reduce any other α,β-unsaturated carbonyl (data not shown), and thus the substrate(s) of *Le*OPR2 remain to be identified. This apparent lack of activity is unlikely to be due to the N-terminal $(His)_6$-tag, as other fusion constructs including *Le*OPR2 carrying either N-terminal GST, or C-terminal $(His)_6$-tags, lacked OPR activity as well (not shown).

The data presented here fully support the conclusions drawn from the phylogenetic analysis (Figure 2): *Le*OPR3 is the putative *At*OPR3 orthologue, i.e. the octadecanoid pathway enzyme for (+)-7-*epi*-JA biosynthesis. *Le*OPR1 is closely related to *At*OPRs 1 and 2, not only in primary structure but also with respect to its biochemical properties (Straßner *et al.*, 1999) and its stereospecificity for 9*R*,13*R*-OPDA. *Le*OPR2 is the most distantly related homologue and this is reflected in the lack of OPR activity and the absence of a bound flavin cofactor.

Subcellular localization of tomato OPRs

The enhanced green fluorescent protein (EGFP) was fused to the N-terminus of *Le*OPR1 and 3, respectively, and the subcellular localization of the fusion proteins was analyzed by confocal laser scanning microscopy after transient expression in onion epidermal cells. This system has been widely used for the analysis of protein routing to various subcellular locations (Hanson and Köhler, 2001). Characteristic cytosolic and nuclear staining was observed when EGFP was expressed alone (Figure 5a,b). Likewise, a diffuse cytosolic staining was observed for the EGFP–OPR1 fusion protein (Figure 5c,d). Cells expressing the EGFP–OPR3 fusion, on the other hand, exhibited punctuate staining in the cytosol indicating the association of OPR3 with organellar structures. The fluorescence of EGFP-OPR3 was found to co-localize with that of red fluorescent protein (RFP) fused to

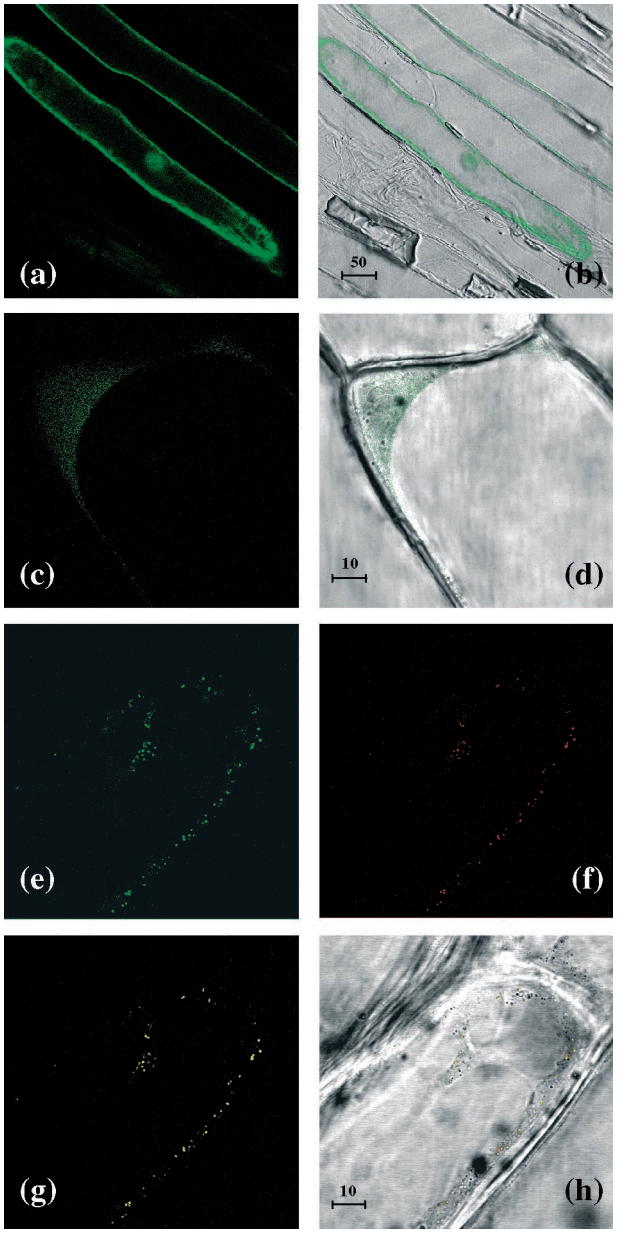

Figure 5. Subcellular localization of tomato OPRs.
The subcellular localization in onion epidermal cells of transiently expressed EGFP–RFP-fusion proteins was analyzed by confocal laser scanning microscopy. The green fluorescence of EGFP expressed alone can be detected in the cytosol and the nucleus (a, b). Likewise, expression of the EGFP–*Le*OPR1 fusion protein results in a diffuse cytosolic staining (c, d). Punctuate staining is observed for the EGFP-*Le*OPR3 and RFP–*So*GOX-fusion proteins (e, f). Merging of the two color channels in (e), and (f) results in a yellow signal, indicating co-localization of the two fusion proteins (g). Overlays of the fluorescent signal in (a, c), and (g) with the corresponding light micrographs is shown in (b, d, and h). The size bar corresponds to 10 or 50 μM, respectively.

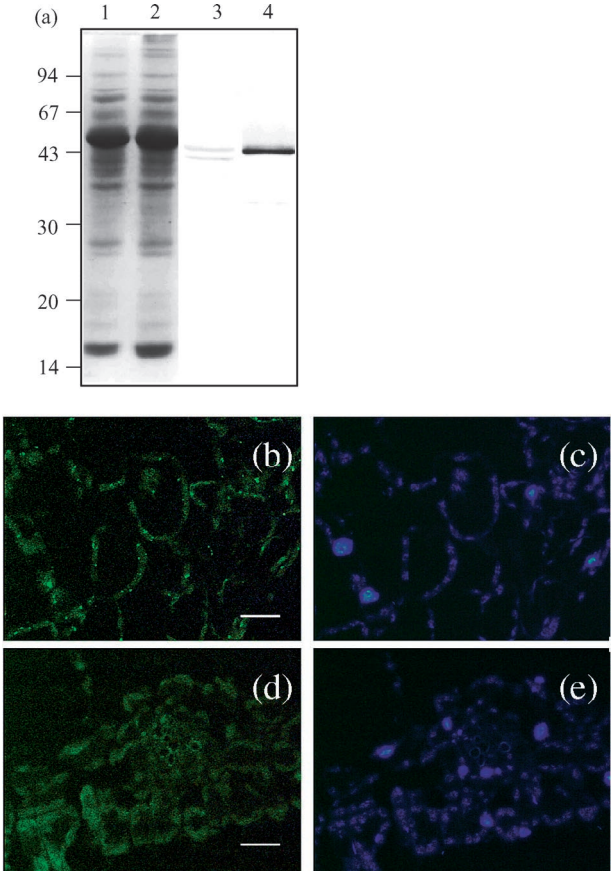

Figure 6. Immunolocalization of *Arabidopsis* OPRs.
(a) A polyclonal antiserum directed against recombinant *At*OPR3 was used for immunolocalization. The specificity of the serum was tested on Western blots. Protein extracts (40 μg) of *opr3* mutant (lanes 1 and 3) and wild-type plants (lanes 2 and 4) were separated by SDS–PAGE and stained with Coomassie brilliant blue (lanes 1 and 2). Proteins were transferred from a duplicate gel to a nitrocellulose membrane (lanes 3 and 4) and immunodecorated using the *At*OPR3 antiserum (1 : 5000).
Cross-sections of *Arabidopsis* leaves were immunodecorated using the *At*OPR3 antiserum. The immunfluorescence is shown on the left for the wild-type *Arabidopsis* (b), and the *opr3* mutant (d), respectively. In *opr3*, diffuse cytosolic staining can be observed (d), while additional, particulate staining is present in the wild-type *Arabidopsis* (b). The identical sections were stained with DAPI for visualization of the nuclear and plastidic DNA (c, e). The size bar corresponds to 10 μM.

glycolate oxidase from spinach (RFP–*So*GOX; Figure 5e–h). Co-localization with a *bona fide* peroxisomal protein identifies the peroxisomes as the subcellular location of *Le*OPR3. Targeting of *Le*OPR3 to the peroxisomes is likely mediated by a three-amino acid carboxy-terminal extension (Ser–Arg–Leu) which constitutes a potential peroxisomal targeting signal (Olsen, 1998) that is present in OPR3 but absent from OPR1 and 2 both in tomato and in *Arabidopsis* (Stintzi and Browse, 2000).

Subcellular localization of Arabidopsis OPRs

In order to confirm the peroxisomal localization of OPR3s also in *Arabidopsis*, a polyclonal antiserum was raised against recombinant *At*OPR3. When tested on protein gel blots of heterologously expressed *Arabidopsis* OPRs, the

antiserum immunodecorated *At*OPR3 and, with a substantially reduced intensity, also *At*OPR1 and 2 (data not shown). The specificity of the antiserum was tested on gel blots of protein extracts from wild-type plants and *Arabidopsis* mutants which, due to a single T-DNA insertion in the *OPR3* gene, do not express the OPR3 protein and lack the capacity to synthesize JA (Sanders *et al.*, 2000; Stintzi and Browse, 2000; Stintzi *et al.*, 2001). The *At*OPR3-antiserum specifically immunodecorated a protein of the size expected for OPR3 in extracts of wild-type plants but not in the *opr3* mutant (Figure 6a). In the latter extract two faint protein bands were detected only after extended development and these protein band may correspond to *At*OPR1 and/or 2 (Figure 6a). The *At*OPR3-antiserum was then used for the immunolocalization of OPRs in cross-sections of *Arabidopsis* leaves. In mesophyll cells of wild-type leaves, intense fluorescence was observed apparently associated with organellar structures, surrounded by a diffuse cytosolic staining (Figure 6b). As evident from the pattern obtained after staining of DNA (Figure 6c), the intense spots of fluorescence were not associated with either nuclei or plastids. In the *opr3* mutant, the same antiserum produced only a weak labeling of cytoplasmic regions (Figure 6d). Therefore, notwithstanding the cross-reactivity of the antiserum with *At*OPRs 1 and 2, we conclude that the intense fluorescent specks in wild-type leaf cross-sections indicate the subcellular location of *At*OPR3 while the diffuse staining is likely caused by *At*OPR1 and/or 2.

In a second approach, sucrose density gradient centrifugation was employed to determine the subcellular site of OPR activity in *Arabidopsis*. In the experiment shown, racemic *cis*-OPDA was used as the substrate and, consequently, the observed activity corresponds to the combined activities of *At*OPRs 1–3. In extracts of wild-type plants, two peaks of OPR activity were observed (Figure 7a). Fractions from the top of the gradient contained the cytosolic activity as well as that released from broken organelles. The second peak of OPR activity co-localized with catalase, a marker enzyme for microbodies, indicating the presence of OPR in peroxisomes (Figure 7a). A comparison of activity profiles obtained for wild-type plants on the one hand, and for the *Arabidopsis opr3* mutant on the other hand clearly identifies the peroxisomal OPR as *At*OPR3. In the activity profile obtained for *opr3* plants, no peak of activity was detected in peroxisomal fractions. This conclusion is supported by the protein gel blot shown in Figure 7(b). Combined antisera against *At*OPR1 and 3 used as the primary antibody immunodecorated a band of the expected size in the sucrose density gradient fractions corresponding to microbodies as well as cytosolic proteins from *Arabidopsis* wild-type plants. In gradients derived from *opr3* plants, the band in high-density fractions was missing. The cumulative evidence demonstrates conclusively that *At*OPR3 protein and activity are localized to the peroxisomes.

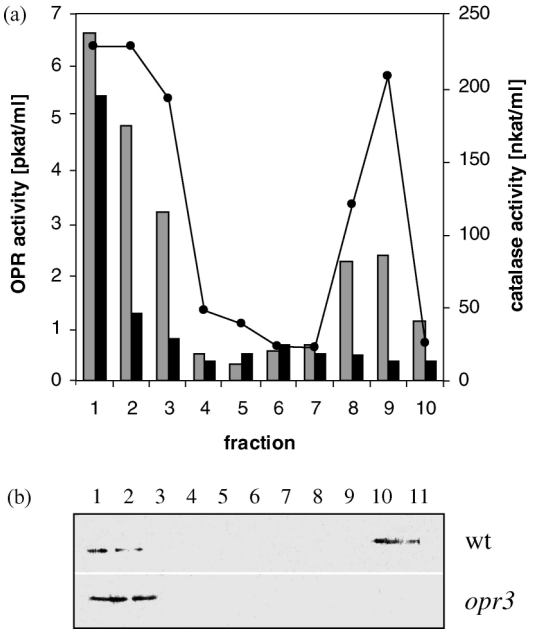

Figure 7. Subcellular fractionation and detection of *Arabidopsis* OPR activity.
(a) Localization of OPR activity. Crude extracts of wild-type *Arabidopsis* (gray bars) and *opr3* mutant (black bars) shoot tissue were fractionated by sucrose density gradient centrifugation. OPR activity was assayed in 200 μl aliquots of the 4 ml fractions using racemic *cis*(+/–)-OPDA as the substrate. Catalase activity (●) was assayed in 50 μl aliquots as a marker enzyme for microbodies in the gradient obtained from *opr3* plants.
(b) Localization of OPR protein. In a further, independent experiment, equal aliquots of sucrose density gradient fractions from wild-type plants (top) and *opr3* mutant plants (bottom) were analyzed on Western blots that were immunodecorated with the combined antisera against *At*OPRs 1 and 3, respectively (1 : 10 000 each).

cDNA-microarray analysis of wound-induced changes in gene expression

In order to analyze the expression of OPR isozymes in a broader context of genes potentially involved in wound signal transduction, a dedicated microarray was generated containing a total of 230 tomato cDNAs. cDNA probes were chosen to represent genes of wound and general stress responses, pathogenesis, proteases and protease inhibitors, aromatic amino acid and phenylpropanoid biosynthesis, primary metabolism, house-keeping and unknown functions, as well as proteins implicated in signal transduction processes. The array also contained 26 genes involved in fatty acid/lipid biosynthesis and metabolism including gene-specific probes for enzymes of oxylipin biosynthesis, i.e. *LOXC* (Heitz *et al.*, 1997), *LOXD* (Heitz *et al.*, 1997), *AOS1* (Sivasankar *et al.*, 2000), *AOS2* (Howe *et al.*, 2000), *AOC* (Ziegler *et al.*, 2000), fatty acid hydroperoxide lyase (*HPL,* Howe *et al.*, 2000), divinylether synthase (*DES*, Itoh and Howe, 2001), and *OPR1–3* (Straßner *et al.*, 1999; this work).

When the transcript levels of wounded and unwounded (control) leaves were compared, a rapid accumulation was observed for the LOXD, AOS1 (to a lesser extent also

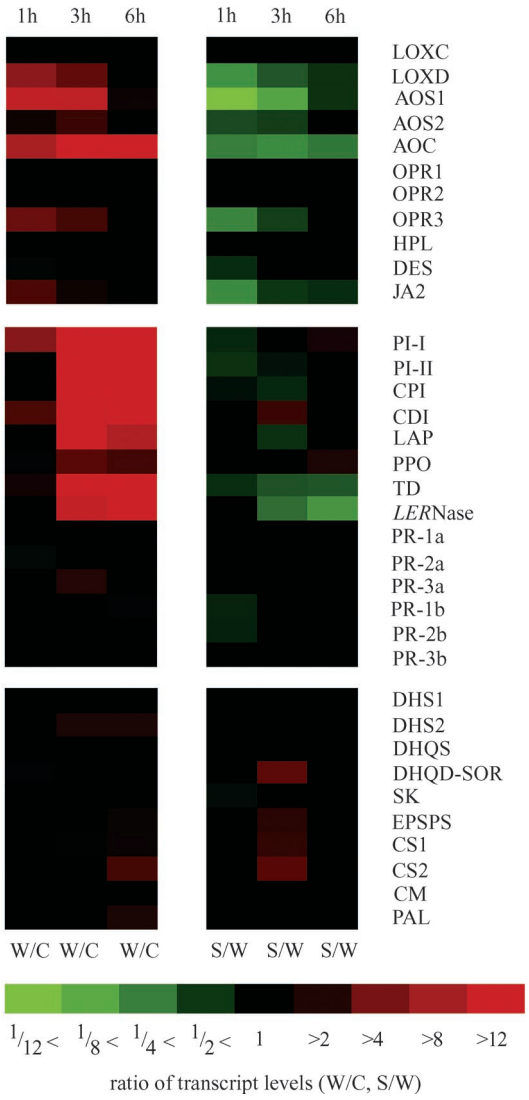

Figure 8. cDNA-microarray expression analysis.
The steady-state transcript levels corresponding to selected cDNAs on the microarray (see 'Experimental procedures') were analyzed 1, 3, and 6 h after wounding. The relative abundance of any one transcript in wounded leaves (W) as compared to the leaves of unwounded control plants (C) is shown on the left (W/C). The right column shows relative transcript abundance (S/W) in apical, unwounded leaves (i.e. systemic leaves, S) in relation to the basal, wounded leaf (W). The three blocks in each column comprise enzymes of oxylipin metabolism (top), systemic wound response proteins and pathogenesis-related proteins (center), and aromatic amino acid biosynthesis (bottom). Transcript ratios greater (smaller) than one are indicated in shadings of red (green) of increasing intensity. A transcript was considered to be enriched (depleted) when the ratio was greater than 2 (smaller than 0.5) in two out of two independent experiments and the data represent the mean of the ratios obtained in the two experiments. The figure was generated using the programs CLUSTER and TREEVIEW (written by M. Eisen, Stanford University; http://rana.stanford.edu/software).

AOS2), and AOC transcripts, which was transient for LOXD and AOS1 and more persistent for AOC (Figure 8). These transcripts represent enzymes specifically involved in the octadecanoid pathway, i.e. that branch of oxylipin metabolism committed to JA biosynthesis. Supporting a role for OPR3 in the octadecanoid pathway, its mRNA concentration showed a comparably rapid and transient increase while the expression levels of OPRs 1 and 2 remained unaffected. Furthermore, the transient induction of *Le*JA2, a NAC-domain transcription factor (Collinge and Boller, 2001; tomato EST clone cLED34B13; accession number AF011555) was observed. Co-regulation of this transcription factor with octadecanoid pathway enzymes may indicate a function in JA signal transduction. No increase in transcript abundance was observed for enzymes in other branches of oxylipin metabolism, i.e. those leading to the production of short-chain aldehydes (HPL) or divinylethers (DES). The early increase in mRNAs of octadecanoid pathway enzymes was succeeded by the high-level accumulation of transcripts for systemic wound-response proteins (SWRPs; probes referenced in Bergey *et al.*, 1996; Constabel *et al.*, 1995; Lers *et al.*, 1998; Schaller *et al.*, 1995) including proteinase inhibitors I and II (PI-I, PI-II), carboxypeptidase inhibitor (CPI), cathepsin D inhibitor (CDI), leucine aminopeptidase (LAP), polyphenol oxidase (PPO), threonine deaminase (TD) and *LE*RNase. Expression levels of pathogenesis-related genes (PR-1a,b – PR-3a,b; probes referenced in Schaller *et al.*, 2000a) remained unaffected.

A comparison of the transcriptome in wounded versus apical, unwounded leaves (Figure 8) revealed comparable levels of SWRP mRNAs in both tissues. Transcipt levels were identical (within a factor of two) in wounded and unwounded leaves of the same plants for PI-I and -II, CDI, CPI, LAP, and PPO. Only the TD and *LE*RNase transcripts were less abundant in unwounded as compared to wounded leaves. In contrast, all mRNAs for octadecanoid pathway enzymes (LOXD, AOS1, AOS2, AOC, OPR3) were of lower abundance in apical, unwounded as compared to the wounded leaves of the same plants. For these transcripts, the fold induction in the wounded versus control leaves was similar in magnitude to the reduction in systemic as compared to wounded tissues, suggesting that the capacity for JA biosynthesis was up-regulated locally but not systemically. The transcripts of some enzymes of aromatic amino acid biosynthesis, on the other hand, accumulated to higher levels in systemic as compared to wounded tissues (i.e. 3-dehydroquinate dehydratase-shikimate–NADPH oxidoreductase (DHQD–SOR), 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS), and chorismate synthase (CS1 and 2), as opposed to DAHP synthase (DHS1 and 2), shikimate kinase (SK), chorismate mutase (CM), and phenylalanine ammonia-lyase (PAL); cDNA probes referenced in Görlach *et al.*, 1995; Bischoff *et al.*, 2001).

RNA gel blot analyses were performed to confirm some of these observations, in particular those related to the expression of *Le*OPR isozymes. Radiolabeled cDNAs identical to the ones included in the microarray were used as hybridization probes, and the specificity for the respective *OPR*

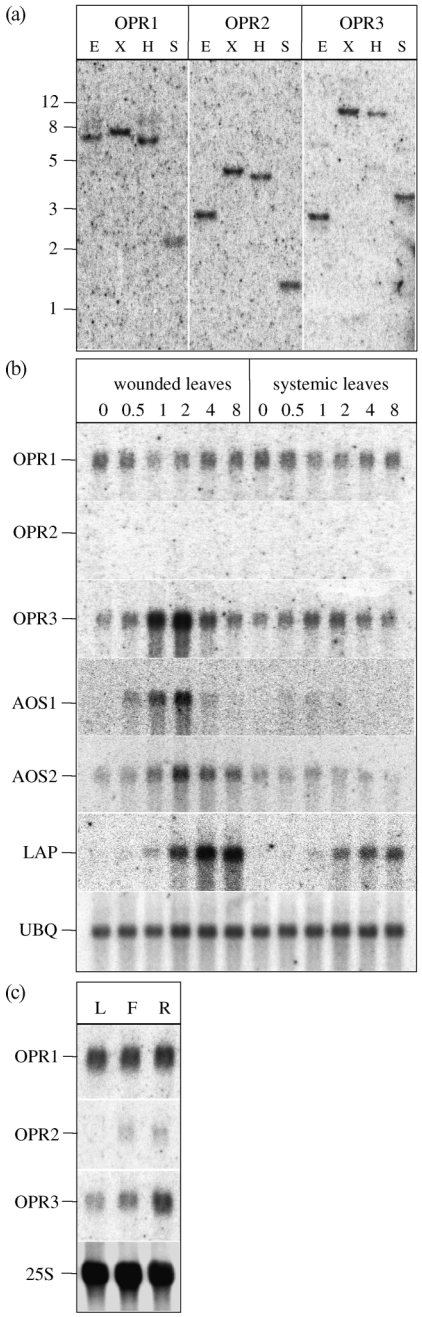

Figure 9. DNA and RNA gel blot analyses.
(a) DNA gel blot analysis. Ten microgram of tomato genomic DNA was restricted with *Eco*RI (E), *Xho*I (X), *Hin*dIII (H), and *Ssp*I (S), and was separated by agarose gel electrophoresis. DNA fragments were transferred to nitrocellulose membranes and the blot was hybridized with radiolabeled PCR products corresponding to 5′- and 3′-untranslated regions of *Le*OPR cDNAs. The position of size standards (1 kb-ladder, GibcoBRL) is indicated. (b, c) RNA gel blot analyses. Total RNA was isolated from the wounded leaves and the apical, unwounded (systemic) leaves of 12 tomato plants at the indicated time points (in h) after wounding (b) as well as from tomato leaves (L), flowers (F), and roots (R) of unwounded plants (c). Five microgram of total RNA was separated on formaldehyde agarose gels and subsequently transferred to nitrocellulose membranes. Radiolabelled DNA fragments of *Le*OPRs (probes were identical to the ones used for the experiments shown in Figures 8 and 9a), leucine aminopeptidase (LAP), polyubiquitin (UBQ), or 25S rRNA were used as hybridization probes.

genes was confirmed by Southern blot analyses (Figure 9a). The abundance of the *Le*OPR1 mRNA was not affected by wounding (Figure 9b), and was found to be similar in leaves, flowers, and roots of tomato plants (Figure 9c). Consistent with the paucity of *Le*OPR2 EST clones in the database (see 'Experimental procedures' section), the corresponding transcript was barely detectable. Transcripts of *Le*OPR3 were less abundant in leaves than in flowers and roots but accumulated in leaves after wounding. The steady-state mRNA levels for *Le*OPR3 continued to rise for two hours in the wounded leaf and declined thereafter. In apical, unwounded leaves, however, very little induction of *Le*OPR3 was observed. Similarly, the AOS1 transcript accumulated locally with kinetics identical to those of OPR3, while an induction in systemic leaf tissue was barely detectable (Figure 9b). For AOS2, a low-level constitutive expression was observed. Also, for this transcript, a transient accumulation was observed in wounded but not in undamaged leaves of wounded plants (Figure 9b). In contrast, the accumulation of LAP transcripts, representing the SWRPs, was delayed in comparison to *Le*OPR3 and was observed in both the wounded and, to a somewhat lower extent, in the systemic tissue (Figure 9b). These results are fully consistent with those obtained in the cDNA-microarray analyses.

Concentration of OPDA/JA

The expression analyses using cDNA microarray and RNA gel blots suggested an up-regulation of the octadecanoid pathway for JA biosynthesis in the wounded but not in the apical, unwounded leaves of wounded tomato plants. To investigate whether or not the observed increase in expression at the transcript level is reflected in a corresponding increase in the biosynthesis of octadecanoids, the contents of OPDA and JA were analyzed in the respective tissues. Methanol extracts were prepared from the lower wounded and the apical, unwounded leaves of 2-week-old tomato plants. Oxylipins were purified by solid-phase extraction, methylated, and analyzed by gas chromatography–mass spectrometry (GC–MS). The leaves of unwounded tomato plants had a relatively high content of OPDA (630 ± 160 pmol g^{-1} FW; Figure 10a). The OPDA concentration remained constant in undamaged leaves of wounded plants. In contrast, 30 min after wounding, the OPDA content increased almost two-fold in the wounded leaves and remained at this elevated level throughout the experiment (6 h, Figure 10a). The JA level, on the other hand, was close to the detection limit prior to wounding, but increased dramatically within 30 min in wounded tissues. As opposed to OPDA, JA accumulated transiently, declining back to basal levels within 3 h. No significant change in JA content was detectable in systemic leaves (Figure 10b).

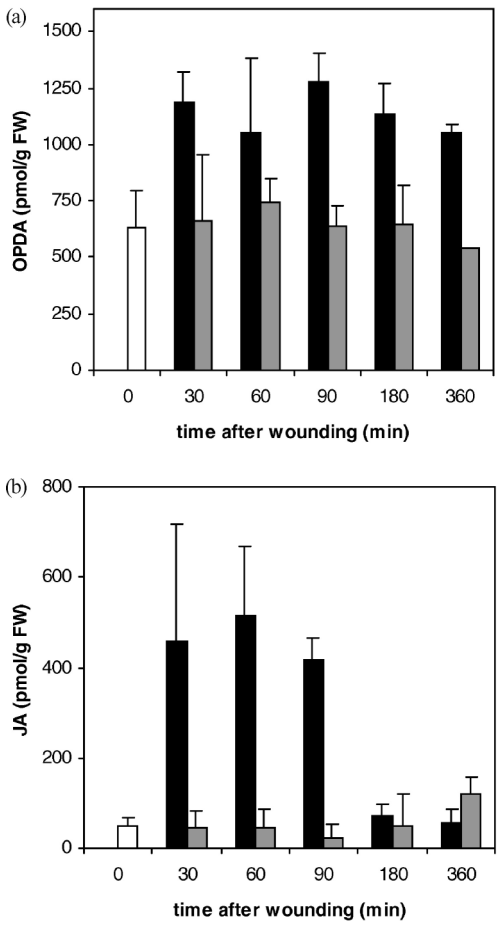

Figure 10. OPDA and JA content in wounded versus apical, unwounded leaves.
The concentrations (in pmol g^{-1} FW) of OPDA (a) and JA (b) were analyzed by GC–MS in methanolic extracts of leaves of unwounded control plants at time zero (white bars), and in the lower wounded (black bars) and upper undamaged leaves (gray bars) of wounded tomato plants at the time points indicated. The data are the mean ± SE of three independent experiments.

Discussion

Two new homologues of 12-oxophytodienote reductases (OPRs) were cloned and characterized from tomato bringing the total number of tomato isozymes up to three. A phylogenetic analysis comparing tomato OPRs with the previously characterized enzymes from *Arabidopsis* revealed a close relationship of *Le*OPR1 with *At*OPRs 1 and 2 and of *Le*OPR3 with *At*OPR3, while *Le*OPR2 appeared to be more distantly related to the *Arabidopsis* enzymes. These relationships were found to be reflected in the substrate specificity and, to some extent, also in the expression pattern and the subcellular localization of OPR isozymes.

Le*OPR2*

*Le*OPR2, heterologously expressed in *E. coli*, differed from the other two OPRs in that it lacked a bound flavin cofactor and OPR activity. Furthermore, the recombinant enzyme carrying either N- or C-terminal affinity tags had neither detectable NADPH oxidase activity, nor did it reduce α,β-unsaturated carbonyls (data not shown). This is in contrast to all other characterized enzymes in this family of FMN-dependent oxidoreductases, i.e. the Oed yellow enzyme (OYE)-family, the name being derived from the prototypical Old Yellow Enzyme from brewer's bottom yeast (Schaller and Weiler, 1997b; Schaller, 2001; Warburg and Christian, 1932). As *Le*OPR2 was not yet expressed in its native form, it cannot be excluded that its lack of activity is in fact caused by the N- or C-terminal affinity tags. Substantial differences exist, however, between *Le*OPR2 and other OPRs with respect to the amino acid side chains that contribute to the active site (not shown), which may provide an alternative explanation for its unusual properties. *Le*OPR2 is expressed at comparatively low levels in tomato roots, leaves, and flowers. Since its endogenous substrates are unknown, the physiological function in these tissues remains obscure. Despite extensive biochemical characterization, this is also true for many other OYEs including the yeast enzymes.

Le*OPR1*

*Le*OPR1 resembles *At*OPRs 1 and 2 not only in primary structure but also with respect to substrate specificity. Both enzymes reduce a broad range of α,β-unsaturated carbonyl compounds (Straßner *et al.*, 1999; F. Schaller, unpublished results) including 9*R*,13*R*-OPDA. 9*S*,13*S*-OPDA, the precursor of biologically active jasmonic acid, on the other hand, is not a substrate of either *Le*OPR1 or *At*OPRs 1 and 2 (Schaller *et al.*, 1998, 2000b; Figure 4). These OPRs are, thus not involved in the octadecanoid pathway, i.e. that branch of oxylipin metabolism which leads to the production of 3*R*,7*S*-JA. This conclusion is consistent with the apparent lack of wound-inducibility of the OPR1 and 2 transcripts in tomato (Figures 8 and 9). In contrast, *Arabidopsis* OPR1 is transcriptionally activated in response to mechanical wounding, touch and wind stimulation, UV-C illumination, and heat or cold stress (Biesgen and Weiler, 1999; Reymond *et al.*, 2000). Yet, the significance of this up-regulation at the transcript level is unclear since no change in either protein abundance or enzyme activity was observed under the respective stress conditions (Biesgen and Weiler, 1999). While *At*OPR1 resembles the octadecanoid pathway enzymes with respect to the wound-inducibility of its transcript, the underlying regulatory mechanisms are clearly different which is indicative of distinct physiological functions: Using the JA-insensitive *coi1* mutant it was shown that the up-regulation of LOX2 and AOS expression in *Arabidopsis* depends on a functional JA-signaling pathway while that of OPR1 does not (Reymond *et al.*, 2000). Furthermore, also in the *opr3* mutant background, the regulation of *OPR1* expression was shown to differ from that of *COI1*-dependent

genes (e.g. *LOX2, AOS*) in that it was induced by application of exogenous OPDA but not by JA (Stintzi *et al.*, 2001).

A function has been suggested for OPRs 1 and 2 in maintaining a stereochemically homogenous pool of 9*S*,13*S*-OPDA by removal of 9*R*,13*R*-OPDA. The undesirable OPDA enantiomer may result *in planta* from spontaneous cyclization of its allene oxide precursor due to a partial uncoupling of the AOS and AOC reactions (Laudert *et al.*, 1997; Ziegler *et al.*, 1997, 2000). Considering the respective subcellular localization of the enzymes involved, such a role seems now unlikely, however: The AOS and AOC reactions are clearly confined to plastids (Froehlich *et al.*, 2001; Howe *et al.*, 2000; Laudert *et al.*, 1996; Sivasankar *et al.*, 2000; Ziegler *et al.*, 2000), while *Le*OPR1 and *At*OPR1 are cytosolic enzymes (Figures 5–7). The physiological substrate of OPR1 is, therefore more likely to be found in the cytosol.

The recent X-ray structure analysis of *Le*OPR1 revealed the structure of the catalytic cavity, and the mode of substrate binding supporting α,β-unsaturated carbonyls as the natural substrates (Breithaupt *et al.*, 2001). In the structure of the enzyme–substrate complex, specific binding of 9*R*,13*R*-OPDA was evident, corroborating the experimentally observed stereoselectivity of the enzyme (Figure 4), and favoring aliphatic α,β-unsaturated carbonyls structurally related to 9*R*,13*R*-OPDA as the physiological substrates (Breithaupt *et al.*, 2001). Such compounds are generated during the oxidative burst in wounded and diseased plant tissue and have cytotoxic as well as gene-inducing activities (Deighton *et al.*, 1999; Vollenweider *et al.*, 2000). The specific induction of *OPR1* gene expression in *Arabidopsis* leaves under oxidative stress conditions (C. Ochsenbein and K. Apel, personal communication), provides some support for the suggested role for OPR1 in the detoxification or modification of these reactive α,β-unsaturated carbonyls (Vollenweider *et al.*, 2000). Participation in the biosynthesis of as yet unidentified oxylipins and modulation of the oxylipin signature remains a further possibility.

Le*OPR3*

Following reports on lipoxygenase (Heitz *et al.*, 1997), alleneoxide synthase (Howe *et al.*, 2000; Sivasankar *et al.*, 2000), and alleneoxide cyclase (Ziegler *et al.*, 2000), the present work on *Le*OPR3 completes the cloning and characterization of enzymes specifically involved in the biosynthesis of JA in tomato. *Le*OPR3 was found to be closely related to *At*OPR3 with respect to primary structure (Figure 2) and substrate specificity, catalyzing the formation of both 9*S*,13*S*- and 9*R*,13*R*-OPC8:0 from the respective OPDA substrates (Figure 4). Using independent techniques, i.e. confocal laser scanning microscopy of transiently expressed GFP fusion proteins, immunolocalization, and subcellular fractionation, we have demonstrated the peroxisomal localization of OPR3 in tomato and *Arabidopsis* (Figures 5–7). Therefore, the octadecanoid pathway appears to be confined to plastids and peroxisomes and, in contrast to previous assumptions (Schaller, 2001), does not involve the cytosolic compartment. The first steps of the pathway catalyzed by LOXD, AOS1, and AOC lead to the synthesis of cyclopentenone signaling molecules within the plastids (Froehlich *et al.*, 2001; Heitz *et al.*, 1997; Sivasankar *et al.*, 2000; Ziegler *et al.*, 2000). The compartmentalization of the reactions resulting in the shortening of the OPC8:0 side chain, presumably catalyzed by the enzymes of β-oxidation (Vick and Zimmerman, 1984), has not been investigated yet. Microbodies are, however, the primary sites of β-oxidation in plants (Beevers, 1979; Gerhardt, 1983), and the final steps of JA biosynthesis are thus generally believed to occur in peroxisomes. The peroxisomal localization of OPR3 which catalyzes the formation of OPC8:0 as a substrate for subsequent β-oxidation, lends support to this hypothesis. The biosynthesis of cyclopentanones and cyclopentenones is thus confined to different subcellular compartments. The apparent spatial separation of cyclopentenones and cyclopentanones may be relevant for the differential activities of the two classes of signaling molecules (cf. Introduction, and the work of Stintzi *et al.*, 2001). It also raises the question of how OPDA, the intermediate, is shuttled from one compartment to the other. Specific transport processes may exist in chloroplast and peroxisome membranes, respectively, and a close association of the two organelles may facilitate a direct transfer avoiding the cytosolic compartment. The recent detection of a significant pool of OPDA in esterified form in the *sn*1-position of plastid galactolipids (Stelmach *et al.*, 2001) opens the further possibility of a direct transition of OPDA between organellar membranes in lipid-bound form.

Future work will also have to focus on the final steps of JA biosynthesis which are believed to include the activation of OPC-8:0 by an acyl-CoA synthetase, followed by peroxisomal β-oxidation catalyzed by three enzymes, i.e. acyl-CoA oxidase, the multifunctional protein, and thiolase. While multiple isoforms exist for each of these enzymes (Germain *et al.*, 2001; Shockey *et al.*, 2000; references therein), it is unclear at this time, whether or not a set of enzymes dedicated to the synthesis of JA exists. If this is the case, then a specialized peroxisome may be envisioned, as the different classes of plant peroxisomes are characterized by their respective complement of matrix enzymes (Olsen, 1998). This notion may be supported by the observation of peroxisome proliferation in wounded *Arabidopsis* plants (Lopez-Huertas *et al.*, 2000). Alternatively, the final steps in JA biosynthsesis may be catalyzed by the enzymes of general fatty acid catabolism.

The role for OPR3 in JA biosynthesis is further supported by the cDNA microarray analysis of gene expression revealing the wound-induced accumulation of *Le*OPR3 transcripts. The coordinate regulation of LOXD, AOS1, AOS2,

AOC, and OPR3 suggests the involvement of these particular isoforms in wound-induced JA biosynthesis. Notwithstanding previous reports on the wound-inducibility of these enzymes in tomato and other species (Bell and Mullet, 1991, 1993; Heitz *et al.*, 1997; Howe *et al.*, 2000; Laudert and Weiler, 1998; Müssig *et al.*, 2000; Reymond *et al.*, 2000; Royo *et al.*, 1996; Sivasankar *et al.*, 2000; Ziegler *et al.*, 2000, 2001), a more comprehensive picture of the coordinated regulation of the pathway emerges from the use of a cDNA microarray containing gene-specific probes. The induction of transcript accumulation for the entire set of octadecanoid pathway enzymes including OPR3 suggests that the formation of cyclopentenones (OPDA) is not sufficient, but rather the production of cyclopentanones (JA) is important for the wound response in tomato plants. This conclusion is in partial agreement with results obtained for *Arabidopsis*, where both OPDA and JA were found to be required for the induction of defense genes to maximum levels. Yet OPDA *per se* caused some activation of defense genes which, under the experimental conditions of this study, was sufficient to provide full resistance to the larvae of *Bradysia impatiens* (Stintzi *et al.*, 2001).

The role of jasmonate synthesis in systemic wound signaling

Tomato plants respond to herbivore feeding and mechanical wounding with the induction of defense gene expression both in the wounded as well as in the apical, unwounded leaves (Green and Ryan, 1972). According to a well-established model of wound signaling, the 18-amino-acid peptide systemin, which is released as a consequence of wounding, causes the receptor-mediated activation of the octadecanoid pathway leading to the production of JA, and, ultimately, defense-gene (*SWRP*-gene) activation (Farmer and Ryan, 1992; Ryan, 2000). The up-regulation of JA biosynthesis and, in turn, defense-gene activation by systemin and its precursor protein prosystemin is supported by a wealth of biochemical and genetic data (reviewed by Ryan, 2000). Most relevant for the following discussion, synthetic systemin is known to induce both the accumulation of JA (Doares *et al.*, 1995) and *SWRP*-gene expression (Schaller *et al.*, 1995). Furthermore, the ectopic expression of a *35S::prosystemin* transgene was shown to result in the generation of a graft-transmissible signal, capable of inducing *SWRP* expression over long distances (McGurl *et al.*, 1994). While the data are most easily reconciled with systemin itself being the long-distance signal, it is equally possible that systemin induces the production of an as yet unidentified systemic signal molecule, thus amplifying the wound response (Ryan, 2000). Our data seem to favor the latter possibility.

Using RNA gel blot and cDNA microarray expression analysis we show here the more rapid and transient accumulation of transcripts for octadecanoid pathway enzymes as compared to systemic wound response proteins (SWRPs), which is consistent with current models of wound signal transduction, according to which two classes of wound-responsive genes exist, i.e. the 'early genes' with signaling function and the 'late genes' coding for proteins with a direct role in herbivore-defense (Ryan, 2000). Surprisingly, however, there was very little induction of octadecanoid pathway transcripts in systemic as compared to wounded tissues, while SWRP transcripts accumulated to comparable levels in both wounded as well as apical, unwounded leaves. This is consistent with the previous observation of limited systemic induction of the AOS transcript in tomato (GAH, Howe *et al.*, 2000; unpublished observation) and in contrast to findings in *Arabidopsis*, where the systemic induction of AOS expression was observed after local wounding (Kubigsteltig *et al.*, 1999; Laudert and Weiler, 1998). Apparently, in tomato systemic tissues, the up-regulation of the JA biosynthetic capacity is not required for defense gene induction.

To corroborate the gene expression data, the levels of OPDA and JA were analyzed in wounded tomato plants. We observed a rapid accumulation in wounded leaves which was transient for JA and more sustained for OPDA. Therefore, at later time points, the accumulation of OPDA appears to be uncoupled from JA biosynthesis. This finding may indicate a regulatory role of OPR3 in controlling the relative levels of OPDA and JA, i.e. two signaling molecules with potentially differing activities (cf. Introduction, and the work of Stintzi *et al.*, 2001). Additional points of control for the regulation of the relative levels of OPDA and JA may reside in the molecules facilitating the transport of OPDA from plastids to peroxisomes. In undamaged leaves of wounded plants, however, the concentration of both OPDA and JA remained unchanged during the 6-h experiment. While there are conflicting reports in literature in favor (Herde *et al.*, 1996; Herde *et al.*, 1999) and against (Bowles, 1998; Rojo *et al.*, 1999; Ziegler *et al.*, 2001) a systemic increase in JA levels after wounding of tomato or *Nicotiana attenuata* plants, our observations are fully consistent with the gene-expression data and suggest differential roles for octadecanoids in wound signaling in local and systemic tissues.

Apparently, the signaling events in wounded and apical, unwounded leaves of the same plant are not identical and the transcriptional activation of the octadecanoid pathway accompanied by the accumulation of JA to high levels is not required for defense gene induction in tomato systemic tissues. This conclusion is in complete agreement with a recent study showing that the perception but not the synthesis of JA is required for the activation of the wound response in systemic tissues of tomato (Li *et al.*, 2002). Reciprocal grafting experiments were performed using tomato mutants that either fail to synthesize (*spr-2*; Howe and Ryan, 1999) or perceive (*jai-1*; Li *et al.*, 2001) the JA

signal molecule. When grafted plants were wounded below the graft junction, the activation of the wound response in the scion depended on the ability to perceive JA. Wound- or (pro)systemin-induced activation of the jasmonate biosynthetic pathway, on the other hand, was required in the lower part of the plant for the generation of a graft-transmissible signal, but not for defense gene activation in the scion (Li *et al.*, 2002). The findings reported here and in the study by Li *et al.* (2002) have important implications for the proposed role of systemin as the systemic wound signaling molecule. Systemin is known to induce the accumulation of both JA and the transcripts of octadecanoid pathway enzymes. We observed neither JA accumulation nor the induction of octadecanoid pathway transcripts in systemic tissues, suggesting that the activity of systemin is confined to the wounded leaf, where it induces the synthesis of OPDA, JA, and possibly other oxylipin signaling molecules which are candidate molecules for the systemic signal in the activation of *SWRP* genes.

Experimental procedures

Cloning of Le*OPR2 and three cDNAs*

Database searches using the *Le*OPR1 cDNA sequence (accession number AJ242551, Straßner *et al.*, 1999) as the query identified six related tomato ESTs, one of which was unique (EST275940; *Le*OPR2), while five appeared to be derived from the same transcript (EST279187, EST268087, EST245043, EST246270, EST286138; *Le*OPR3). All EST clones appeared to be incomplete at their 5′-ends. RACE-PCR using the SMART RACE cDNA amplification kit (Clontech; Palo Alto, CA) was performed according to the manufacturer's instructions to obtain the full-length cDNAs of *Le*OPR2 (AJ278331) and *Le*OPR3 (AJ278332). In a first step, single-stranded cDNA was synthesized from total RNA of tomato leaves using M-MLV reverse transcriptase (Promega; Madison, WI) and oligo(dT) as the primer. Subsequently, the full-length cDNAs were amplified using primers (Microsynth; Balgach, Switzerland) corresponding to the 3′-ends of the *Le*OPR2 and *Le* OPR3 open-reading frames (*Le*OPR2: 5′-ATATACATGTGAATAATTAGGAAGCAACTT-3′; *Le*OPR3: 5′-AACGTTTCTTCTCACAGACGCGATAACGG-3′) and the universal primer provided with the kit. The PCR products were gel-purified and cloned into the vector pCR2.1-TOPO (Invitrogen; Groningen, the Netherlands). The identity of the cDNA sequences was confirmed by sequence analysis of at least three independent PCR products using fluorescent dideoxy chain terminators in the cycle sequencing reaction (Perkin-Elmer; Foster City, CA) and the Applied Biosystems model 373 A DNA sequencer.

RNA and DNA gel blot analyses

Tomato plants (*Lycopersicon esculentum* cv. Castlemart II; Ochoa Seed Company, Gilroy, CA) were grown for 14 days as described (Schaller *et al.*, 2000a). Plants were wounded twice at a terminal leaflet with a pair of tweezers and leaf tissue was harvested at the time points indicated from wounded leaves, apical unwounded leaves, and leaves of unwounded control plants. For RNA gel blot analysis, 4.5 μg samples of total RNA extracted from tomato tissues were separated on formaldehyde–agarose gels and transferred to nitrocellulose membranes. Likewise, genomic DNA was isolated from tomato leaves (Nucleon Phytopure DNA extraction kit; Amersham Pharmacia Biotech, Dübendorf, Switzerland), digested with the enzymes indicated in the figure legends, separated by agarose gel electrophoresis, and DNA fragments were transferred to nitrocellulose membranes. RNA and DNA gel blots were hybridized with radiolabeled cDNA probes, the blots were washed and evaluated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) as described (Schaller and Oecking, 1999).

cDNA microarray analysis of gene expression

A dedicated cDNA microarray was generated containing 233 DNA fragments (230 tomato cDNAs, 3 fragments of fungal genomic DNA as negative controls) representing wound response proteins (28), pathogenesis-related proteins (10), proteases and proteinase inhibitors (43), oxylipin biosynthetic enzymes (15), proteins of aromatic amino acid and phenylpropanoid biosynthesis (17), proteins with potential roles in signal transduction with special emphasis on protein kinases (29), proteins of primary metabolism and house-keeping function (40), as well as cDNAs of diverse and unknown functions that had been isolated serendipitously in various screens over the years. Many of the cDNA clones had been obtained as ESTs from Clemson University Genomics Institute (Clemson, SC), others had been under investigation in the laboratories of AS and GAH (a complete list of the cDNA clones used can be obtained at http://www.pbp.ipw.biol.ethz.ch/projects_schaller.htm). The identity of all EST clones was verified by sequence analysis. DNA fragments were amplified by PCR as described (Reymond *et al.*, 2000) using primers complementary to vector sequences flanking the cDNA inserts. Alternatively, when gene-specific probes were required, the 3′- or 5′-untranslated regions of the respective cDNAs were amplified using gene-specific and vector-derived primers. Quality and quantity of the PCR products were controlled by agarose gel electrophoresis. PCR products were purified by ethanol precipitation, resuspended in 3 × SSC, and spotted on SuperAldehyde microscopic slides as described (Reymond *et al.*, 2000).

For the generation of fluorescent probes, 2 μg poly(A)+ RNA was purified using the Oligtex Midi kit (Qiagen; Basel, Switzerland) and reverse-transcribed in the presence of Cy3-dCTP or Cy5-dCTP (Amersham Pharmacia Biotech). Published protocols were followed for reverse-transcription, purification of probes, as well as hybridization and washing of the arrays (Reymond *et al.*, 2000, available at http://www.unil.ch/ibpv). Separate scans were performed for each fluorophor using a GSM 418 Array Scanner (Genetic MicroSystems, Paul Bucher; Rotkreuz, Switzerland). The photomultiplier settings were adjusted in each channel to result in a just saturated signal for the most highly expressed genes. The signal intensities, as the mean pixel intensity within a given spot, and expression ratios were calculated using the ImaGene software (BioDiscovery) assuming a ratio of 1.0 as the average over non-induced clones on the array for normalization. For the Cy5 fluorophor, the background signal intensity in the area surrounding the spotted cDNAs was often higher than that of poorly expressed genes. Therefore, to avoid negative values, we did not subtract the background signal. Thus, the actual induction/repression factors for transcripts of low abundance may be slightly higher than the values presented. Expression ratios were log-transformed to result in numerically identical values for induction and repression of equal magnitude. Induction and repression were defined as a minimum of a two-fold change in the expression ratio in two out of two independent experiments. In control experiments using two identical RNA samples, a Cy5/Cy3 signal ratio of 1.0 should

ideally result for each spot in the array after data normalization. For 98% of the 233 clones, the observed ratio was within a factor of 1.5 of the expected value, supporting the significance of a two-fold change in the expression ratio (not shown).

Expression in E. coli *and purification of* Le*OPR1–3*

*Le*OPR1 was expressed as an N-terminal glutathione *S*-transferase (GST) fusion protein in *E. coli* and purified from bacterial extracts by affinity chromatography on glutathione–Sepharose 4B (Amersham Pharmacia Biotech) as described (Straßner *et al.*, 1999). Expression constructs for *Le*OPR2 and 3 were generated by PCR using the respective cDNAs as the template and *Pwo* DNA polymerase (Roche Diagnostics; Rotkreuz, Switzerland) in the amplification reaction. The primers for *Le*OPR2 (5′-CTAGCTAGCCACCACCACCACCACCACCACATGGAAGCAAACTCTAACTCTGCTGTG-3′, 5′-CCGCTCGAGTTAGGAAGCAACTTCAAGAAATGG-3′) and *Le*OPR3 (5′-CTAGCTAGCCACCACCACCACCACCACATGGCGTCTTCAGCTCAAGATGGAA-3′, 5′-CCGCTCGAGTTCTCACAGACGCGATAACGGTC-3′) comprised *Nhe*I and *Xho*I restriction sites to facilitate the directional cloning into the expression vector pET21a (Novagen; Madison, WI), and 6 His-codons for an amino-terminal affinity tag facilitating the purification of the recombinant proteins. The expression constructs were transformed into *E. coli* BL21 codon plus (DE3)-RIL (Stratagene, LaJolla, CA) and cultures were grown to an OD600 of 1.0 at 37°C. The expression of recombinant proteins was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to 1 mM. After 4 h at 30°C, cells were harvested by centrifugation (18 000 *g* for 15 min at 4°C). The cells were resuspended in 50 mM sodium phosphate buffer pH 8.0, 300 mM NaCl (500 mM ammonium sulfate in case of OPR3), 10 mM imidazole, 0.1 mg ml^{-1} *DNa*sel, 1 mg ml^{-1} lysozyme, 1 mM PMSF, and 4 mM benzamidine. After 20 min at room temperature, cells were lysed by sonication. The cell debris was removed by centrifugation (10 000 *g* for 30 min at 4°C), and the supernatant was subjected to affinity chromatography on Ni–NTA agarose according to the manufacturer's (Qiagen, Basel, Switzerland) recommendations.

Production of OPDA and assay of OPR stereospecificity

Racemic *cis*-OPDA was produced enzymatically from linolenic assay as described (Laudert *et al.*, 1997). Optically pure 9*S*,13*S*-OPDA *(cis*(+)-OPDA) and 9*R*,13*R*-OPDA *(cis*(–)-OPDA) were purified from the racemic mixture by preparative HPLC (Schaller *et al.*, 2000b). For assay of stereospecificity, racemic *cis*-OPDA (0.1 mM) was used as substrate of recombinant *Le*OPR isoforms (0.75 μg) in 0.5 ml 50 mM potassium phosphate buffer (pH 7.5) in presence of 1 mM NADPH. After 30 min at 25°C, substrate consumption and product formation were analyzed by capillary GC–MS as described (Laudert *et al.*, 1997; Schaller *et al.*, 1998).

OPDA and JA extraction

The tomato plants used were of the same age and developmental stage as those for RNA gel blot and cDNA microarray expression analyses. The procedure for OPDA and JA analysis was adapted from Laudert *et al.* (1997). About 0.25 g of leaf material (lower wounded and apical unwounded leaves) was harvested at the time points indicated after wounding, extracted for 15 min at 55°C in 1.5 ml methanol containing 500 pmol mg FW^{-1} of [$^2H_5$]-*cis*-(+/–)-OPDA (Laudert and Weiler, 1998) and 100 pmol mg FW^{-1} of [$^{13}C_2$]-JA (gift of Axel Müller, Ruhr-Universität Bochum, Germany) as internal standards. The extract was evaporated under vacuum to dryness. The residue was dissolved in 200 μl diethyl ether, cleared by centrifugation, and passed through an aminopropyl solid-phase Chromabond–NH_2 extraction column (Macherey Nagel, Hoerdt, France). The column was washed twice with 150 μl chloroform:2-propanol (2 : 1, v/v) and jasmonates were eluted in two volumes of 200 μl diethyl ether:formic acid (98 : 2, v/v). Eluates were evaporated to dryness under a stream of nitrogen, dissolved in 50 μl of methanol, methylated with 100 μl ethereal diazomethane, and dried again. The material was finally redissolved in 8 μl chloroform and analyzed by GC–MS.

Gas chromatography–mass spectrometry

All measurement were performed on a Varian Saturn 2000 ion-trap mass spectrometer operated in CI-MRM mode (chemical ionisation, methanol) with the following settings: GC, splitless injection of 1 μl (injector temp. 260°C) using a ZB-50 fused silica capillary column (30 m, 9.25 mm i.d., 0.25 μm film thickness; Phenomenex, Aschaffenburg, Germany) with a He carrier gas flow of 1 ml ml^{-1}; chromatographic conditions: 1 min 50°C, linear ramp at 20°C min^{-1} to −250°C; transfer line 260°C; mass spectrometric conditions: 2 scans sec^{-1} (100–200 amu). Analyzing both the *cis* and the *trans* isomers, endogenous JA was detected by collecting $m/z = 225$ [M + H] + of JA methyl ester ($R_t = 10.05$; 10.25 min) and observing the fragment ion $m/z = 207$. The internal standard [^{13}C]-JA was determined by analyzing the corresponding ions $m/z = 227$ and 209 ($R_t = 19.05$ min). The JA concentration was derived from the signal ratio of the unlabelled $m/z = 207$ over the isotopically labeled mass fragment $m/z = 209$. Similarly, OPDA was analyzed by detecting the product ions $m/z = 275$ and 280 from the precursors $m/z = 307$ (OPDA) and $m/z = 312$ ([$^2H_5$]-OPDA) at $R_t = 15.31$ and 14.5 min, respectively.

Constructs for transient expression of EGFP–RFP-fusion proteins

Constructs were generated for the transient expression of *Le*OPR1, *Le*OPR3, and *Spinacia oleracea* glycolate oxidase (*So*GOX) in N-terminal fusion with either EGFP (Clontech) or RFP (Contech) in onion epidermal cells under the control of the CaMV 35S promoter.

- EGFP–OPR1: The expression vector pSH11, a derivative of bluescript pSK (Stratagene) containing an expression cassette consisting of the 35S promoter and the *NOS* terminator for expression of EGFP (Clontech), was the kind gift of Dr J. Bauer (ETH Zürich). The open reading frame (ORF) of *Le*OPR1 was amplified by PCR using Pfu Turbo DNA polymerase (Stratagene) and synthetic oligonucleotide primers (5′-AAGGAAAAAAGCGGCCGCGATGGAAAATAAAGTCGTTGAAGAG-3′, 5′-GCAATGCATTGGTTCTGCAGTCATGTCATGGTTTCTAGAAATGG-3′). The PCR product was cloned downstream of the EGFP ORF using *Not*I and *Pst*I restriction sites. The joined ORFs encode a fusion protein comprising EGFP and *Le*OPR1 at its N- and C-termini, respectively.
- EGFP–OPR3: The *Le*OPR3 ORF was amplified by PCR using synthetic oligonucleotide primers (5′-AAGGAAAAAAGCGGCCGCGATGGCGTCTTCAGCTCAAGATGG-3′, 5′-CCAATGCATTGGTTCTGCAGTCACAGACGCGATAACGGTCCATTGC-3′) and cloned into pSH11 as described above.
- RFP–GOX: Synthetic oligonucleotide primers containing *Not*I and *Bam*HI restriction sites (5′-CGCGGATCCCGCCACCATGAGGTCTTCCAAGAATG-3′, 5′-GAATTCTAGAGTCGCGGCCGCATAAAGGAACAGATGGTG-3′) were used in the PCR to amplify the ORF of RFP including the Kozak translational initiation site

from pDsRed (Clontech). The PCR product was digested with *Bam*HI and *Not*I to replace the respective EGFP-encoding fragment in pSH11. Subsequently, the ORF of *So*GOX amplified by PCR from a cDNA clone (accession number J03492) kindly provided by A. Baker (University of Leeds) was joined to the 3′-end of the RFP ORF by cloning into the *Not*I site (forward primer: 5′-AAGGAAAAAAAGCGGCCGCGATGGAGATCACAAATGTGAACGAG-3′, reverse primer: 5′-TTTCCTTTTGCGGCCGCGTTATAATCTGGCAACAGGACCAG-3′). The identities of all expression constructs were verified by DNA sequence analysis.

Transient expression and subcellular localization of GFP–RFP-fusion proteins

The transient expression system in onion epidermal cells (Scott *et al.*, 1999) was used to analyze the subcellular targeting of the EGRP and RFP fusion proteins by confocal laser scanning microscopy. Onion epidermal strips were placed on agar plates containing MS medium and the expression constructs were delivered into the cells using a particle inflow gun (Vain *et al.*, 1993; 4 μg of plasmid DNA and 4 μg of gold particles per shot). Cells were allowed to recover for 24–48 h before they were analyzed by confocal laser scanning microscopy using an ArKr laser at 488 nm. GFP and RFP fluorescence were recorded between 503 and 550 nm and 560–640 nm, respectively (Leica TCS SP, Leica DM IRBE; Leica Microsystems, Wetzlar, Germany).

Immunolocalization of Arabidopsis *OPRs*

For the generation of a polyclonal antiserum, recombinant *At*OPR3 was purified by affinity chromatography and SDS–PAGE as described (Schaller *et al.*, 2000b) and electroeluted from the respective gel slice. Five consecutive immunizations of a rabbit were performed intradermally with 0.1 mg of protein in Freund's incomplete adjuvant at 1-week intervals. After 2 weeks, 0.1 mg were injected intramuscularly. The polyclonal serum was obtained from two bleeds, 7 and 14 days thereafter. For protein gel blot analyses, the serum was diluted 1 : 5000- or 1 : 10 000-fold, a goat-antirabbit–IgG alkaline phosphatase-conjugate (Bio-Rad) was used as the secondary antibody and bromochloroindolyl phosphate–nitro blue tetrazolium were used as chromogenic substrates. For immuno-histological experiments, the serum was diluted 2500-fold and used on *Arabidopsis* wild-type (Col-0) and *opr3* mutant leaf tissue, fixed, cut, and embedded as described by Van Lammeren *et al.* (1985) using goat-antirabbit-IgG fluorescein-5-isothiocyanate (FITC)-conjugate (Sigma) as the secondary antibody. DNA was stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, 1 mg ml^{-1} in phosphate-buffered saline).

Subcellular fractionation and localization of Arabidopsis *OPR activity*

Arabidopsis wild-type plants (ecotype Col-0) and *opr3* mutant plants (ecotype Wassilewskija; Sanders *et al.*, 2000) were grown under short day conditions for 7–9 weeks. Shoot tissue of 30 plants was harvested and homogenized in 20 ml 0.15 M Tricine buffer, pH 7.5, 1 mM EDTA, 10 mM KCl, 1 mM $MgCl_2$, 10 mM Na_2SO_4, 0.1% (w/v) BSA, and 12% (w/v) sucrose. The homogenate was filtered through four layers of cheese cloth and 10 ml of the filtrate was loaded onto a sucrose density gradient (30–60%, 24 ml). After centrifugation (61 000 *g*, 3 h, 4°C), the gradients were fractionated and fractions (4 ml) were assayed for OPR and catalase activities. OPR activity in 0.2 ml of the fractions was assayed for 30 min at 25°C in a total volume of 1 ml potassium phosphate buffer (pH 7.5) containing 0.1 mM racemic *cis*-OPDA and 1 mM NADPH. Reaction products were quantified by capillary GC–MS (Schaller and Weiler, 1997a). For the assay of catalase activity as a marker for microbodies, 0.05 ml of the sucrose gradient fractions were added to 0.1 M potassium phosphate buffer (pH 7.0), 10 mM H_2O_2 in a total volume of 1 ml and the consumption of H_2O_2 was followed spectrophotometrically at 240 nm ($\epsilon = 3.6 \times 10^4\ cm^2\ mol^{-1}$).

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