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Jasmonate biosynthesis and the allene oxide cyclase family of *Arabidopsis thaliana*

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Abstract

In biosynthesis of octadecanoids and jasmonate (JA), the naturally occurring enantiomer is established in a step catalysed by the gene cloned recently from tomato as a single-copy gene (Ziegler *et al.*, 2000). Based on sequence homology, four full-length cDNAs were isolated from *Arabidopsis thaliana* ecotype Columbia coding for proteins with AOC activity. The expression of *AOC* genes was transiently and differentially up-regulated upon wounding both locally and systemically and was induced by JA treatment. In contrast, AOC protein appeared at constitutively high basal levels and was slightly increased by the treatments. Immunohistochemical analyses revealed abundant occurrence of AOC protein as well as of the preceding enzymes in octadecanoid biosynthesis, lipoxygenase (LOX) and allene oxide synthase (AOS), in fully developed tissues, but much less so in 7-day old leaf tissues. Metabolic profiling data of free and esterified polyunsaturated fatty acids and lipid peroxidation products including JA and octadecanoids in wild-type leaves and the jasmonate-deficient mutant OPDA reductase 3 (*opr3*) revealed preferential activity of the AOS branch within the LOX pathway. 13-LOX products occurred predominantly as esterified derivatives, and all 13-hydroperoxy derivatives were below the detection limits. There was a constitutive high level of free 12-oxo-phytodienoic acid (OPDA) in untreated wild-type and *opr3* leaves, but an undetectable expression of *AOC*. Upon wounding *opr3* leaves exhibited only low expression of *AOC*, wounded wild-type leaves, however, accumulated JA and AOC mRNA. These and further data suggest regulation of JA biosynthesis by OPDA compartmentalization and a positive feedback by JA during leaf development.

Abbreviations: α-LeA, α-linolenic acid; AOC, allene oxide cyclase; *cet*, mutant with constitutive expression of thionin; *dad1*, mutant with delayed anther dehiscence1; dn-OPDA, dinor-12-oxo-phytodienoic acid; 13-HPOT, 13*S*-hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic acid; JA, jasmonic acid; JAME, JA methyl ester; L.A, linoleic acid; LOX, lipoxygenase; OPDA, 12-oxo-phytodienoic acid; *opr3*, mutant defective in OPR3; OPR3, OPDA reductase3; PLA1, phospholipase of the A1 type; PUFA, polyunsaturated fatty acids; SA, salicylate

Introduction

Generation of lipid-derived signal molecules is a common phenomenon in higher organisms. In plants jasmonates and octadecanoids are of particular importance (Bergey *et al.*, 1996; Ryan, 2000). They originate from polyunsaturated fatty acids (PUFA) and are formed by one of the seven different branches of the LOX pathway, the AOS branch (Feussner and Wasternack, 2002). The other branches lead to leaf aldehydes and leaf alcohols as well as various derivatives of PUFAs such as epoxy-, hydroxy-, keto- or ether PUFA and epoxy hydroxy PUFA (Feussner and Wasternack, 2002).

The nucleotide sequence data reported will appear in the EMBL GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers AJ308483 (*AOC1*); AJ308484 (*AOC2*); AJ308485 (*AOC3*) and AJ308486 (*AOC4*).

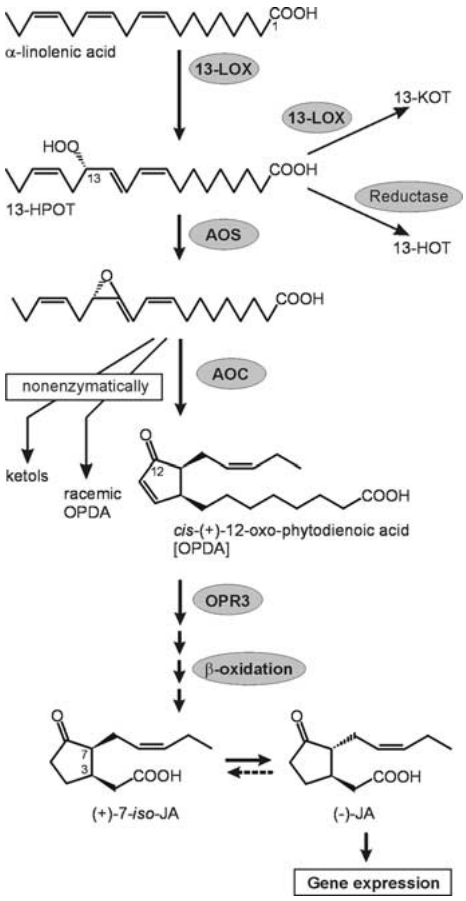

Figure 1. Scheme of JA biosynthesis and further 13-LOX products derived from α-LeA, 13-HPOT and (9*Z*,11*E*,15*Z*)-13-keto-(9,11,15)-octadecatrienoic acid (13-KOT). Identical reactions occur with LA as substrate, whereas the corresponding 9-derivatives are formed via 9-LOX catalysis.

The biosynthesis of jasmonic acid (JA) and its methyl ester (JAME) was elucidated by Vick and Zimmerman (1983) and Hamberg and Hughes (1988). With α-linolenic acid (α-LeA) as substrate, molecular oxygen is inserted by a 13-LOX at carbon atom 13 leading to the formation of a fatty acid hydroperoxide 13*S*-hydroperoxy-(*9Z*,11*E*,15)-octadecatrienoic acid, 13-HPOT (Figure 1). This compound is dehydrated by the allene oxide synthase (AOS) to an unstable allene oxide which can be either hydrolysed non-enzymatically to α- and γ-ketols or cyclized to racemic 12-oxo-phytodienoic acid (OPDA). In the presence of an allene oxide cyclase (AOC), preferential formation of the (9*S*,13*S*) enantiomer of OPDA occurs. The AOC-catalysed step is regarded as the crucial step in octadecanoid and jasmonate biosynthesis because only this enantiomeric form is the substrate for the naturally occurring (+)-7-*iso*-JA, which is formed after reduction of OPDA by a specific OPDA reductase (OPR3) and three cycles of β-oxidation (Schaller *et al.*, 2000; Ziegler *et al.*, 2000).

Several cDNAs coding for LOXs, AOSs and OPRs have been cloned from different plant species as reviewed by Schaller (2001) and Feussner and Wasternack (2002). The first AOC was recently cloned from tomato and found to be encoded by a single-copy gene (Ziegler *et al.*, 2000). Treatment of plants with products of the AOS branch, the octadecanoids and jasmonates, led to accumulation of mRNAs coding for LOX, AOS, AOC and OPR3 suggesting a feed-forward regulation in JA biosynthesis (Sasaki *et al.*, 2001). Also, biotic or abiotic stresses, which result in endogenous increases of octadecanoids and jasmonates, are usually accompanied by a transcriptional up-regulation of AOS, AOC and OPR3 (Howe *et al.*, 2000; Maucher *et al.*, 2000; Ziegler *et al.*, 2000). However, the function of this mRNA accumulation is not understood, because it was not accompanied by endogenous formation of jasmonates as shown by isotopic dilution analysis using barley and tomato leaves during the first 24 h of mRNA accumulation (Kramell *et al.*, 2000; Miersch and Wasternack, 2000). Furthermore, the rise in JA upon wounding appeared before the onset of AOS or AOC mRNA accumulation (Ziegler *et al.*, 2001; Stenzel, 2002).

A chloroplast location of the first half of biosynthetic steps to JA is generally assumed. Since chloroplast LOXs are predominantly localized within the stroma and the inner envelope membranes of chloroplasts (Feussner *et al.*, 1995; Blée and Joyard, 1996), α-LeA of chloroplast envelope membranes is suggested as the substrate for jasmonate biosynthesis. Indeed, the recently identified jasmonate-deficient mutant *dad1* (delayed anther dehiscence1) is defective in a chloroplast-located phospholipase of the A1 type (PLA1) (Ishiguro *et al.*, 2001). Furthermore, it seems ubiquitous that the location of 13-LOXs, AOSs and AOC is the chloroplast, since the proteins were detected in the chloroplast by import studies (Bell *et al.*, 1995; Froehlich *et al.*, 2001), by chloroplast isolation (Harms *et al.*, 1995; Feussner *et al.*, 1995) or by immunocytochemical analysis (Feussner *et al.*, 1995; Ziegler *et al.*, 2000). Even the barley AOS lacking a transit sequence for chloroplast import was immunocytochemically shown to be located within the chloroplast (Maucher *et al.*, 2000). By contrast, OPR3 carries a peroxisomal target sequence (Stintzi and Browse, 2000), and might thus be co-localized

with the subsequent β-oxidation steps in the peroxisomes (Kindl, 1987). It is still unclear, however, how metabolites in JA biosynthesis are channelled between the two compartments involved (Laudert and Weiler, 1998; Froehlich *et al.*, 2001).

In *Arabidopsis*, *AOS* (Kubigsteltig *et al.*, 1999) and *OPR3* (Sanders *et al.*, 2000; Stintzi and Browse, 2000) were shown to be expressed at distinct stages of flower development. Two mutants defective in OPR3 (*dde1*, Sanders *et al.*, 2000; *opr3*, Stintzi and Browse, 2000) and the *dad1* mutant are male-sterile. In leaves of the *opr3* mutant, OPDA accumulated upon wounding, and plants were resistant to fungal and insect attack (Stintzi *et al.*, 2001). Thus, this mutant is a powerful tool to dissect distinct signalling properties of OPDA and JA which have been suggested by several groups (Blechert *et al.*, 1999; Kramell *et al.*, 2000; Vollenweider *et al.*, 2000). Furthermore, *opr3* plants seem to be a useful tool to analyse regulation of JA biosynthesis.

In *Arabidopsis* cDNAs for all enzymes of JA biosynthesis have been cloned, except for AOC and those of β-oxidation. To date, two different LOX cDNAs have been described (AtLOX2, Bell and Mullet, 1993; AtLOX1, Melan *et al.*, 1993). Recently, four other LOX cDNAs were listed in the databases (AtLOX3, AAF79461; AtLOX4, AAF21176; AtLOX5, CAC19365; AtLOX6, AAG52309). Out of these six LOXs, four might be involved in JA biosynthesis (AtLOX2, 3, 4, 6) (Bell *et al.*, 1995; I. Feussner, unpublished). For AOS, a single-copy gene was found (Laudert *et al.*, 1996). Of the three different OPRs, only the recently cloned OPR3 exhibited the exclusive formation of the naturally occurring cyclopentanone compound (Müssig *et al.*, 2000; Sanders *et al.*, 2000; Schaller *et al.*, 2000).

The unique role of AOC in generating the correct enantiomeric form in JA biosynthesis, and its specific occurrence in all vascular bundles and in flower tissues of tomato (Hause *et al.*, 2000a) prompted us to analyse AOC(s) of *Arabidopsis*.

Here, we describe cloning and characterization of four cDNAs coding for proteins with AOC activity. Northern blot analysis revealed transient and differential expression upon wounding. However, AOC, LOX as well as AOS protein appeared constitutively in fully developed leaf tissues with only minor additional accumulation upon wounding. Untreated wild-type and *opr3* leaves exhibited a constitutive high level of free OPDA, but undetectable expression of *AOC*. Upon wounding *opr3* leaves exhibited only low expression of *AOC*; wounded wild-type leaves, however, accumulated JA and AOC mRNA. These and further data on profiles of free and esterified PUFAs and oxylipins suggest regulation of JA biosynthesis by OPDA compartmentalization and positive feedback during growth.

Material and methods

Materials

Opr3 mutant seeds (in the Wassilewskija background) were kindly provided by Prof. J. Browse and Dr A. Stintzi. All non-isotopic jasmonates and octadecanoids were prepared or purchased, checked on purity and used as described (Kramell *et al.*, 2000; Hause *et al.*, 2000a). (13*S*)-HPOT was prepared from α-LeA by incubation with soybean LOX (Sigma, St. Louis, MO). Other hydroperoxy and hydroxy PUFAs as well as other oxylipins were prepared or purchased as described (Kohlmann *et al.*, 1999; Weichert *et al.*, 1999).

The [$^2H_6$]-JA was synthesized as described (Miersch, 1991). [$^2H_5$]-OPDA was prepared from [17-$^2H_2$,18-$^2H_3$]-linolenic acid (Zimmerman and Feng, 1978), and both compounds were used as internal standards in GC-MS analysis. Rabbit polyclonal antibodies against the lipid body LOX of cucumber (Hause *et al.*, 2000b), the recombinant AOS of *A. thaliana* (Laudert and Weiler, 1998) and the recombinant AOC2 of *A. thaliana* were used.

Plant growth and treatments

A. thaliana ecotype Columbia and ecotype Wassilewskija were cultivated in controlled chambers (Percival, CLF) at 70% relative humidity under short-day conditions of 8 h light,

210 μE m^{-2} s^{-1} for 6 weeks. For treatments the complete rosette of a plant was cut above the roots and floated on distilled water, 50 μM JAME, 50 μM 13-HPOT, 1 M sorbitol, 0.5 M glucose, 50 μM salicylate (SA) or 1 M NaCl for indicated times. Wounding was performed by crushing the leaves with a forceps across the mid-vein.

cDNA isolation, RT-PCR, cloning and sequencing

The cDNA library prepared from 4-week old rosette leaves of ecotype Columbia treated for 24 h with 100 μM JAME and maintained in γZAP express Stratagene was kindly provided by Dr Stefan Bau

(Cologne). The cDNA library was hybridized with the full-length AOC cDNA from tomato (Ziegler *et al.*, 2000) (55 °C, 5× SSC, 5× Denhardt's reagent, 100 μg/ml shared salmon sperm DNA, 0.1% w/v SDS, 1×10^6 dpm ml^{-1} of labelled probe, 18 h). Positive clones were isolated and converted into phagemids by *in vivo* excision, and were sequenced on both strands with IRO 700 labelled primers with the Thermo Sequenase DYEnamic Direct Cycle Sequencing Kit (Amersham Pharmacia Biotech). Only one AOC homologue of 991 bp was isolated and designated as *AOC1*.

Based on three other database sequences with partial homology to the *AOC* sequence, we used RT-PCR for isolation of full-length cDNAs. mRNA isolated from rosette leaves floated on 100 μM JAME for 2 h was used with the following primer combinations: AOC2, 5′ primer CCAATCAAGTAGAGTTTCTTC, 3′ primer ACACAGCGATACGAGAAAC; AOC3, 5′ primer CCATTAAAACCAAAGATCAATC, 3′ primer CTAACCTCACACAACTCATC; AOC4, 5′ primer ATTAAACTAACTCAGGCAAG, 3′ primer CCATGACAAAGACACCCAC. Generated RT-PCR products were cloned into the vector pCR II TOPO (Invitrogen) and sequenced.

RNA and immunoblot analysis

Total RNA was extracted from frozen tissues with buffer (4 M guanidinium isothiocyanate, 50 mM sodium citrate pH 4.0, 0.5% sarcosyl, 0.1% 2-mercaptoethanol) and purified by treatments with buffered phenol/chloroform/isoamyl alcohol 25:24:1 (v/v/v). After ethanol precipitations 20 μg per lane was subjected to RNA gel blot analysis according to Sambrook *et al.* (1989). Gel loading was checked by comparing ethidium bromide-stained rRNAs. Hybridization was performed at 60 °C for 16 h with ^{32}P-labelled full-length cDNAs of *AOC1* (Figure 6) or of AOC3 (Figures 5 and 9), and *AOS* of *A. thaliana*. For the creation of gene-specific probes used in Figure 5, we amplified 150 bp of the 3′-untranslated region (3′-UTR) starting after the stop codon of each *AOC*. These PCR products were cloned into the pCR II-TOPO vector and sequenced. The specificity of each probe was tested with PCR-amplified genomic fragments which contained the complete sequence as well as 150 bp of the 5′-UTR and 3′-UTR of each AOC. Gel loading was checked by comparing ethidium bromide-stained rRNAs. Proteins were isolated from the phenolic phase and were used for separation and immunoblot analysis as described by Hause *et al.* (1996, 2000a). Immunoblot analysis was performed by using an antibody raised against recombinant AOC2 (*A. thaliana*) (1:5000 dilution) or against recombinant AOS (*A. thaliana*) (1:2500 dilution) or against cucumber lipid body LOX (Hause *et al.*, 2000b) (1:1000 dilution).

AOC expression in E. coli *and AOC activity assay*

Expression of *AOC1*, *AOC2*, *AOC3* and *AOC4* was performed after cloning of amplified PCR fragments for each AOC as follows. For over-expression of *AOC1*, a fragment covering amino acids 31–253 was generated with the 5′ primer CGC GGATCCC$_{159}$TTGGTTTCTCTAAATCCTT (*Bam*HI restriction site underlined) and the 3′ primer ACCGGT CGACC$_{835}$ACTAATTTGTAAAGTTGCTT AC (*Sal*I restriction site underlined). After cloning into the pCR II-TOPO vector and sequencing, the 680 bp fragment was subcloned into pQE30 with the *Bam*HI/*Sal*I restriction sites. For over-expression of *AOC2*, *AOC3* and *AOC4*, the same approach was used with CGCGGATCCC$_{139}$TTGGTTCCTCTAAATCCTT as 5′ primer and ACCGGTCGACA$_{812}$ATTAGTTGGTA TAgTTACTTAT as 3′ primer for *AOC2*, CGCGGAT CC$_{169}$TTGGTTTCTCAAGATCTTTC as 5′ primer and ACCGGTCGACA$_{851}$CTTAATTAGTAAAGTTA CTTAT as 3′ primer for *AOC3*, and, finally, CGCGGATCCC$_{114}$ TCGGTTTCTCTAGATCCTT as 5′ primer and ACCGGTCGACT$_{790}$TTCAATTAGTAA AGTTAGCGAT as 3′ primer for *AOC4*. pQE30, without or with these inserts, were transformed into the host strain *E. coli* M15. Total protein of isopropyl-β-thiogalactopyranoside (IPTG)-induced or non-induced cultures were isolated and purified as described (Maucher *et al.*, 2000). The resulting supernatant was used for AOC activity assay as described (Ziegler *et al.*, 1997, 1999, 2000).

Quantitative measurement of α-linolenic acid, jasmonates, octadecanoids and other LOX-derived compounds

For quantitative analysis of OPDA, dinor-12-oxo-phytodienoic acid (dn-OPDA) and JA, 1 g (fresh weight) of plant tissue was frozen with liquid nitrogen, homogenized in a mortar and extracted with 10 ml of methanol, and 100 ng of [$^2H_6$]-JA and 100 ng of [$^2H_5$]-OPDA were added as internal standards. The filtrate was loaded onto 3 ml of DEAE-Sephadex A25 columns (Ac$^-$ form, methanol), and the columns

were washed with 3 ml of methanol. After subsequent washing with 3 ml of 0.1 M acetic acid in methanol, eluents obtained with 3 ml of 1 M acetic acid in methanol and with 3 ml of 1.5 M acetic acid in methanol were collected, evaporated and separated on preparative HPLC (column: Eurospher 100-C18, 5 μm, 250 mm $\times$ 4 mm, flow rate 1 ml/min). Separation was performed with solvent A (methanol) and solvent B (0.2% acetic acid in H_2O) with a gradient of 40% solvent A to 100% within 25 min. Fractions eluting between 12 and 13.3 min, between 18.30 and 20 min and between 20.30 and 22 min were collected and evaporated. For subsequent derivatization samples were dissolved in a mixture of 200 μl $CHCl_3$ and *N*,*N*-diisopropylethylamine (1:1, v/v) and derivatized with 10 μl of pentafluorobenzyl bromide at 20 °C overnight. The samples were evaporated, dissolved in 5 ml of *n*-hexane and passed through a SiOH column (500 mg; Machery-Nagel). The pentafluorobenzyl esters were eluted with a mixture of 7 ml of *n*-hexane and diethyl ether (2:1, v/v). After evaporation probes were dissolved in 100 μl of acetonitrile and subjected to GC-MS analysis with a GCQ Finnigan instrument in the following conditions: 70 eV, NCI, ionization gas NH_3, source temperature 140 °C, column Rtx-5w/Integra Guard (Restek, Germany), 5 m inert precolumn; 30 m $\times$ 0.25 mm, 0.25 μm film thickness, injection temperature 250 °C, interface temperature 275 °C; helium 40 cm/s; splitless injection; column temperature program: 1 min at 100 °C, 25 °C/min to 200 °C, 5 °C/min to 300 °C, 20 min at 300 °C. Retention times were for [$^2H_6$]-JA-pentafluorobenzyl ester 11.92 min, for JA-pentafluorobenzyl ester 11.98 min, for dn-OPDA-pentafluorobenzyl ester 18.59 min, for [$^2H_5$]-OPDA-pentafluorobenzyl ester 21.31 min, and for OPDA-pentafluorobenzyl ester 21.39 min. Fragments *m/z* 209 (JA), 215 (JA-standard), 291 (OPDA), 296 (OPDA-standard) and 263 (dn-OPDA) were used for quantification.

PUFA and oxylipins were extracted, derivatized and separated into the corresponding positional and stereoisomeric forms by the following methods. Oxidized fatty acids were extracted according to the method of Weichert *et al.* (1999); for esterified derivatives 0.5 g fresh weight (f.w.) and for free fatty acid derivatives 1.0 g f.w. of frozen leaf tissue was added to 10 ml of extraction solvent (isohexane/isopropanol, 3:2 v/v with 0.0025% w/v BHT) and immediately homogenized with an Ultra Turrax under a stream of argon on ice for 30 s. The extract was centrifuged at 4500 $\times$ *g* at 4 °C for 10 min. The clear upper phase was collected and the pellet extracted three times with 3 ml each of extraction solvent. To the combined organic phases a 6.7% w/v solution of potassium sulfate was added to a volume of 47 ml. After vigorous shaking the upper hexane-rich layer was removed. The upper organic phase containing the oxylipin and fatty acid derivatives was dried under nitrogen and redissolved in 1.5 ml of isohexane/2-propanol (100:5 v/v), and stored under argon at -80 °C until use.

For the analysis of esterified fatty acids, the solvent was removed, and 333 μl of a mixture of toluene and methanol (1:1 v/v) and 167 μl of 0.5 mM sodium methoxide was added. As internal standards triheptadecanoate and triricinoleate were added (100 μg per probe). After incubation of the samples for 20 min, 0.5 ml of 1 M sodium chloride and 50 μl of HCl (37% v/v) were added, and fatty acid methyl esters were extracted twice each with 0.75 ml of hexane. The combined organic phases were evaporated to dryness under a nitrogen stream and the corresponding fatty acid methyl esters were reconstituted in 50 μl of methanol/water/acetic acid (85:15:0.1, v/v).

For the analysis of non-esterified oxylipins in the isohexane/isopropanol extract, the solvent was removed and the sample was solved in 400 μl of methanol. As internal standards heptadecanoic acid and (15*S*,11*Z*,13*E*)-15-hydroxy-11,13-eicosadienoic acid were added. Then 10 μl of an EDAC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide) solution (1 mg EDAC in 10 μl methanol) was added and incubated for 2 h. After adding 200 μl of 0.1 M Tris-HCl, pH 7.5, the fatty acid methyl esters were extracted twice each with 1 ml of hexane. The combined organic phases were evaporated to dryness under a nitrogen stream and the corresponding fatty acid methyl esters were reconstituted in 50 μl of methanol/water/acetic acid (85:15:0.1, v/v).

Oxylipins were analysed by HPLC on an Agilent 1100 HPLC system coupled to a diode array detector (Weichert *et al.*, 1999). At first oxylipins were purified on reversed-phase HPLC. This was carried out on a ET250/2 Nucleosil 120-5 C18 column (Machery-Nagel, 2.1 mm $\times$ 250 mm, 5 μm particle size) with methanol/water/acetic acid (85:15:0.1, v/v) as solvent system, at a flow rate of 0.18 ml/min. Straight-phase HPLC of the hydro(pero)xy fatty acids was carried out on a Zorbax Rx-SIL column (Agilent, 2.1 mm $\times$ 150 mm, 5 μm particle size) with *n*-hexane/2-propanol/acetic acid (100:1:0.1, v/v) as solvent system, at a flow rate of 0.1 ml/min. Chiral-phase HPLC of the hydro(pero)xy fatty acids was carried out on a

Chiralcel OD-H column (Daicel, 2.1 mm × 150 mm, 5 μm particle size) with *n*-hexane/2-propanol/acetic acid (100:5:0.1, v/v) as solvent system, at a flow rate of 0.1 ml/min. Absorbance at 234 nm was monitored. All oxylipins were identified by comparison to the elution times of authentic standards.

Analysis of fatty acid derivatives was carried out by gas chromatography (GC). GC analysis was performed with an Agilent GC 6890 system coupled with a FID detector and equipped with a capillary HP INNOWAX column (0.32 mm × 30 m; 0.5 μm coating thickness; Agilent, Germany). Helium was used as carrier gas (30 cm/s). The samples were measured with a split of 60:1 at an injector temperature of 220 °C. The temperature gradient was 150 °C for 1 min, 150–200 °C at 15 °C/min, 200–250 °C at 2 °C/min, and 250 °C for 10 min. All fatty acids were identified by comparison with the retention times of authentic standards.

Immunocytochemistry

Immunocytochemical analysis was performed as described recently (Hause *et al.*, 2000a). Cross-sections of leaves (2 μm thickness) were immuno-labelled with anti-AOC2 antibody (*A. thaliana*) (diluted 1:2000), anti-LOX antibody (cucumber, diluted 1:500) or anti-AOS antibody (*A. thaliana*, diluted 1:2000). As a secondary antibody a goat anti-rabbit IgG conjugated with Alexa 488 (Molecular Probes, Eugene, OR) was used in the dilution 1:500. Each immunocytochemical staining was analysed under an epifluorescence microscope (Axioskop, Carl Zeiss, Jena, Germany) with the proper filter combination.

Results

Cloning of four cDNAs coding for allene oxide cyclase: primary sequence and gene structure

To identify the *A. thaliana* homologue(s) of the recently cloned tomato AOC (Ziegler *et al.*, 2000), sequence database information revealed existence of four homologous genes. Full-length cDNAs were isolated by screening a λZAP expression library made from mRNA of rosette leaves treated with 100 μM JAME for 24 h, or were generated via RT-PCR from mRNA of rosette leaves floated on 100 μM JAME for 2 h. For this, specific primers for the putative AOC homologues found in the sequence database were used. The four different full-length cDNAs were designated as *AOC1*, *AOC2*, *AOC3* and *AOC4*. The open reading frames ranged from 759 to 774 bp (Table 1), corresponding to proteins containing 253–258 amino acid residues. The calculated molecular masses for all AOCs were about 28 kDa. Using full-length sequences, a neutral pI was calculated for AOC2, whereas for AOC1, AOC3 and AOC4 an alkaline pI was found. In contrast, calculations with sequences lacking the putative chloroplast signal peptide revealed an acidic pI for AOC1 and AOC2, whereas for AOC3 and AOC4 an alkaline pI was calculated (Table 1).

Table 1. Molecular characteristics of AOC1, AOC2, AOC3 and AOC4 of *A. thaliana* ecotype Columbia.

	AOC1	AOC2	AOC3	AOC4
ORF (bp)	762	759	774	759
Number of amino acids	254	253	258	253
Molecular mass (kDa)	27.8	27.6	28.4	27.7
pI (full-length)	9.11	6.91	9.19	9.27
pI (without putative chloroplast target sequence)	5.94	5.4	8.7	8.37
Location on chromosome	3	3	3	1

For activity assays, 5′-truncated cDNAs were cloned into the pQE30 vector and expressed in *E. coli*. Induction of expression in bacteria led to the appearance of an additional band at 26 kDa, which was absent in control bacteria transformed with the empty vector only (data not shown). Examination of bacterial extracts on AOC activity was performed as described (Ziegler *et al.*, 1997, 1999). The assay, which included recombinant AOS protein of barley as a helper enzyme (Maucher *et al.*, 2000) revealed *cis*-(+)-OPDA formation with AOC1, AOC2, AOC3 and AOC4, with the highest activity for AOC2 (data not shown). Given the specificity of the assay conditions, the *cis*-(+)-OPDA formation is indicative of AOC activity. In each AOC sequence of *Arabidopsis*, N-glycosylation sites, phosphorylation sites for protein kinase C, tyrosine kinase and casein kinase II, as well as N-myristolation sites were identified by computional analysis, but their functionality has not been tested yet. A common feature of AOC1, AOC2, AOC3 and AOC4 is the occurrence of a putative chloroplast transit peptide. All N-terminal regions are rich in Ser residues (26–30% in the first 50 amino acids of AOC1–AOC4), the start Met is followed by an Ala residue, and among the first 10 amino acids there is no charged

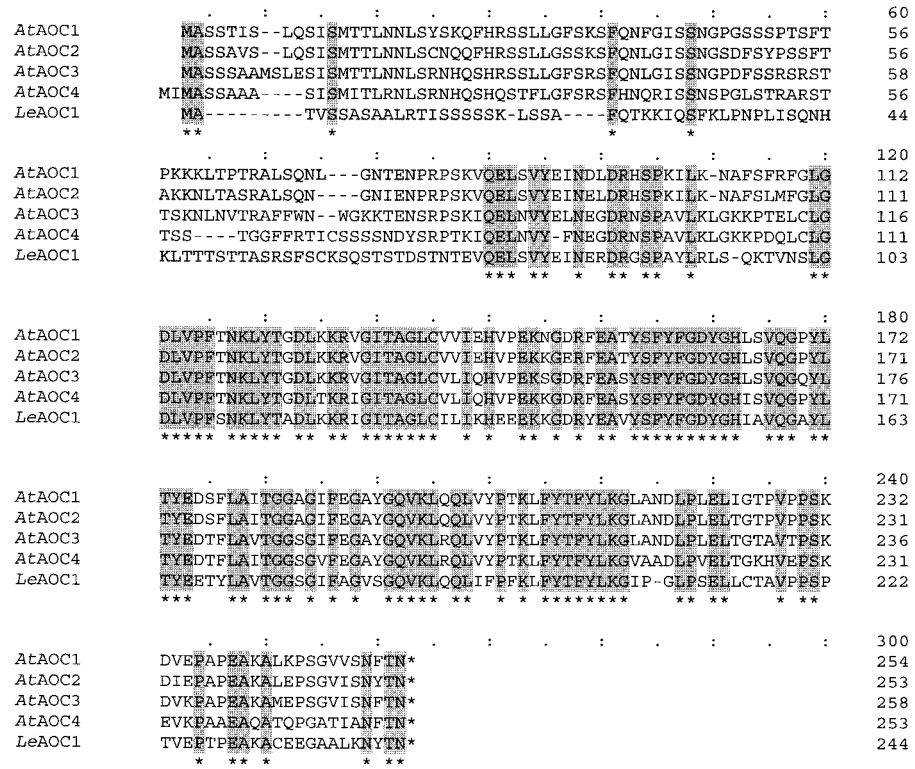

Figure 2. Amino acid sequence comparison of the four isolated AOCs from *A. thaliana* ecotype Columbia with that of tomato *Lycopersicon esculentum* cv. Lukullus published recently (Ziegler *et al.*, 2000). Putative chloroplast transit peptides are boxed in light grey. Conserved sequences are boxed in dark-grey and marked with asterisks.

amino acid. Computer analysis of the first 100 amino acids was performed with the ChloroP V 1.1 program (http://www.dtu.dk/services/ChloroP) (Emanuelsson *et al.*, 1999) and the TargetP program ver. 1.0 (http://www.cbs.dtu.dk/services/TargetP) (Emanuelsson *et al.*, 2000). In both analyses a chloroplast localization was predicted. The putative cleavage site lies between the amino acids 78 and 54 for AOC1, AOC2, AOC3 and AOC4 (Figure 2, shaded in light grey). Using an anti-AOC2 antibody (see below), we confirmed the localization of the AOC protein in chloroplasts by immunocytological analysis. Cross-sections of untreated rosette leaves incubated with anti-AOC2 antibody exhibited a strong green fluorescence label within chloroplasts (Figure 3B, D) indicating the occurrence of AOC protein. After incubation with preimmune serum only yellow-brown autofluorescence was observed (Figure 3A).

Comparison of the cDNA sequences of AOC1, AOC2, AOC3 and AOC4 with sequence database information (http://www.ucbi.nih.gov/) revealed mapping and genomic structure as follows (Figure 4A): *AOC1*, *AOC2* and *AOC3* are located on chromosome 3, whereas *AOC4* is located on chromosome 1. All four coding regions contain one intron of 369 bp, 103 bp, 271 bp and 164 bp, respectively. A search with AOC1 protein sequence with the tblastn program in the EMBL EST database (http://www.ncbi.nlm.nih.gov/blast/) (Altschul *et al.*, 1997) and in the TIGR database (http://www.tigr.org/tab/e2K1/agi/ath1) (Altschul *et al.*, 1990) 35 putative AOC sequences could be found and reconstructed, respectively. Sequence comparison was performed which did not include putative chloroplast signal sequences in the conserved region starting at KVYEL up to the C-terminus. Among the four AOCs an identity of 74–94% was found between AOC2, AOC3 and AOC4, whereas AOC1 exhibited 60–74% identity to all other AOCs. For phylogenetic tree analysis the program Phylip 3.6 (http://www.evolution.genetics.washington.edu/phylip.html) was used (Figure 4B). The AOCs of *A. thaliana* fall into their own subgroup like solanaceous AOCs. The monocotyledonous AOCs are grouped in

a clearly different branch. Gene duplications might have occurred in the case of *A. thaliana*, *Zea mays*, *Physcomitrella patens* and *Gossypium arboretum*.

In order to record differential expression for *AOC1*, *AOC2*, *AOC3* and *AOC4*, we used specific primers amplified 150 bp after the stop codon of the corresponding 3′-UTR of each AOC. Specificity of the primers were found, with 1, 10 and 100 ng genomic PCR products of each AOC which contained the corresponding gene and 150 bp of each 5′- and 3′-UTR (Figure 5A). In the local and systemic wound response of rosette leaves AOC2 mRNA accumulated significantly more than that of AOC1, AOC3 and AOC4. Furthermore, the systemic response in terms of increase in mRNA accumulation was stronger for AOC1 than for the AOC3 and AOC4. Beside these preferential mRNA accumulations, each AOC exhibited transiently a local and systemic mRNA accumulation.

Abundant occurrence of AOC protein in leaves is independent of stress-induced AOC mRNA accumulation

Accumulation of jasmonates and octadecanoids, occurring upon sorbitol treatment (Bohlmann *et al.*, 1998) or wounding (Laudert and Weiler, 1998; Reymond *et al.*, 2000) of *A. thaliana* leaves, correlates in timing with the mRNA accumulation of 13-LOX (Bell *et al.*, 1995) and of AOS (Laudert and Weiler, 1998). We complemented these data by recording accumulation of AOC mRNA. The full-length cDNA (AOC1) which hybridized also with all other mRNAs coding for AOCs was used. Total RNA of rosette leaves treated with JAME, its precursor 13-HPOT or various stresses was probed (Figure 6A). Rapid and transient AOC mRNA accumulation occurred already in detached water-treated rosette leaves, reflecting a wound response by detachment. In contrast, untreated leaves of intact plants exhibited a negligible AOC mRNA accumulation (Figure 8C). Treatment with 50 μM JAME or 13-HPOT (Figure 6A) led to additional AOC mRNA accumulation, which was similar upon treatment with OPDA or JA-amino acid conjugates (data not shown). In case of 13-HPOT treatment, 3-fold higher levels of OPDA and JA could be detected within 30 min (data not shown), indicating endogenous formation of both compounds. Accumulation of AOC mRNA occurred also in a similar amount and time range, when leaves were floated on 1 M sorbitol or 0.5 M glucose. In contrast, with 50 μM SA and, in a more pronounced fashion, with 1 M NaCl an inhibitory effect on AOC mRNA accumulation compared to water-treated leaves was found. For comparison, AOS mRNA accumulation was analysed. Except for NaCl treatment, similar if not identical results were found.

The AOC mRNA levels were not reflected by the AOC protein levels. In most treatments, the AOC protein exhibited constitutively high level with slight rise in the first hour and a significant rise after 8 h (Figure 6B). The anti-AOC2 antibody was able to recognize all four recombinant AOCs (Figure 6C). However, the AOC2 protein was preferentially recognized (cf. legend to Figure 6C). Therefore, AOC2 might be preferentially detected in the immunoblot analysis and the immunocytochemical analysis. Immunocytological inspection revealed an abundant appearance of AOC protein in all tissues of fully developed rosette leaves of the ecotype Columbia, whereas developing rosette leaves showed less AOC protein (Figure 7). A similar distribution and abundance were detected for LOX and AOS at the protein level. This constitutive occurrence of LOX, AOS and AOC protein in untreated fully developed leaf tissues is in apparent contrast to the stress-induced transient accumulation of their mRNAs (Figure 6A) (Bell *et al.*, 1995; Laudert and Weiler, 1998).

The oxylipin profiles of untreated rosette leaves reveal constitutive LOX pathway reactions with preferential activity of the AOS branch

The abundant occurrence of LOX, AOS and AOC proteins in untreated leaf tissues prompted us to determine profiles of fatty acids and various LOX-derived metabolites including the initial JA-precursor 13-HPOT. Each of them was recorded quantitatively for the ecotype Columbia in the free and the esterified form (Table 2). The *Arabidopsis* genome contains no gene coding for divinyl ether synthase. Therefore, formation of divinyl ethers is not to be expected. The amounts of C6 volatiles as well as α- and γ-ketols were below the detection limit which is in the range of 10 pmol per gram f.w. for all compounds tested. In the case of esterified fatty acids a strong preponderance of polyenoic fatty acids was observed with preferential occurrence of α-LeA up to the micromolar range (Table 2). Among esterified compounds originating from LOX activity, substantial amounts (20 nmol/g f.w.) of 13-HOT were detected, whereas other LOX products were found esterified to less than 10-fold lower levels.

Table 2. Amounts of free and esterified fatty acids and the corresponding hydroxy derivatives as well as of free dn-OPDA, OPDA, and JA in untreated 6-week old rosette leaves of ecotype Columbia. Leaf tissue was colleted from 3 to 4 different plants and subjected to extraction and separation as described in Materials and methods.

Fatty acid	Esterified, pmol per gram f.w.	Free, pmol per gram f.w.
16:0	620 000	222 000
16:1	60 000	n.d.[a]
16:3	990 000	n.d.
18:0	50 000	280 000
18:1	180 000	4 000
18:2	900 000	7 000
18:3	2 820 000	10 000
9-HOD	100 000	30
13-HOD[d]	400 000	32
13-/9-KOD	n.d.	10
9-KOD	n.d.	12
9-HOT	300	28
13-HOT	20	179
13-/9-KOT	2 200	n.d.
12-HOT	n.d.	n.d.
16-HOT	n.d.	n.d.
OPDA	–[c]	951
dn-OPDA	–[c]	75
JA	–	43

[a]Not detectable; [b]13-KOD ((9*Z*,11*E*)-13-keto-(9,11)-octadecadienoic acid); [c]Not determined; [d](13*S*,9*Z*,11*E*,15*Z*)-hydroxy-(9,11,15)-octadecadienoic acid.

Among the free fatty acids 16:0 and 18:0 dominated, whereas linoleic acid (LA) and α-LeA were found at 7 and 10 nmol per gram f.w., respectively. The substrate for generation of dn-OPDA, the 16:3 fatty acid, could not be detected in its free form. Also products of the LOX reaction itself, HPOD originating from LA and HPOT originating from α-LeA, could not be detected, although these hydroperoxy compounds are stable at least in part in our work-up procedure (Feussner *et al.*, 1997). However, HOD and HOT derivatives, both indicative of the reductase branch of the LOX pathway, were detected with preference to 13-HOT. LA derivatives accumulated to more than 6-fold lower level without preference of a positional isomer. Among all free LOX-derived products, compounds of the AOS branch were dominant. Nearly 1 nmol per gram f.w. OPDA was found, whereas for dn-OPDA and JA 13-fold and 22-fold lower levels were detected, respectively.

Oxylipin profiles and AOC expression in opr3 mutant leaves suggest a positive feedback loop in JA biosynthesis

The abundant occurrence of OPDA and the 10-fold higher level of the LOX substrate, α-LeA, as well as constitutive high levels of LOX, AOS and AOC protein in untreated rosette leaves raise the question of how regulation of JA biosynthesis occurs. To dissect the effects of JA and OPDA, we performed metabolic profiling and expression analyses of JA biosynthetic genes with untreated and wounded leaves of the JA-deficient mutant *opr3* as well as the corresponding wild-type Wassilewskija. In the case of free and esterified fatty acids, as well as hydroxy and hydroperoxy fatty acid derivatives, most levels were similar if not identical in the *opr3* mutant compared to the wild type. Only 13-HOT levels were 5-fold lower in the mutant, suggesting decreased capacity of the LOX-catalysed step (data not shown).

In untreated leaves of the ecotype Wassilewskija about 2.9 nmol per gram f.w. OPDA was found, which is somewhat above the range of previously detected levels (Stelmach *et al.*, 1999; Reymond *et al.*, 2000; Stintzi and Browse, 2000). This amount exceeds that of JA and dn-OPDA 13- to 35-fold, respectively (Figure 8A). OPDA is also the dominant compound in untreated *opr3* mutant leaves. Despite these remarkable OPDA levels in untreated leaves of both the wild type and the mutant, and the fact that AOC expression is OPDA responsive, AOC mRNA accumulation was below the detection limit (Figure 8C). Upon wounding of wild-type leaves, no dramatic changes occurred in OPDA and dn-OPDA levels compared to a 24-fold increase in JA levels within the first 1.5 h. Even in the *opr3* mutant, OPDA levels did not increase dramatically. The JA level decreased up to 24 h upon wounding of wild-type leaves, whereas OPDA and dn-OPDA levels increased in this time. AOC mRNA and AOS mRNA accumulated abundantly within 1.5 h upon wounding of wild-type leaves (Figure 6A, Figure 8C), possibly caused by the preferential accumulation of JA. Indeed, in wounded leaves of the *opr3* mutant exhibiting the expected JA deficiency (Figure 8B), much less AOC mRNA and AOS mRNA accumulated than in the wild type during 1.5 h of wounding (Figure 8C). Most interestingly, the level of AOC protein, but not of AOS protein, was much less in the untreated *opr3* mutant leaves and increased slightly upon wounding (Figure 8C). This is also indicated by the immunocytochemical inspection, where AOC protein but not

LOX and AOS protein was less abundant in untreated *opr3* mutant leaves than in untreated leaves of the corresponding wild type Wassilewskija (Figure 7). These data suggest a lower capacity of the AOS branch in the JA-deficient mutant *opr3*.

Discussion

In Arabidopsis *four genes encode chloroplast-located AOCs*

The lipid-derived jasmonates and octadecanoids are of considerable and increasing interest by virtue of their unique role in plant stress responses and in distinct stages of development (for review, see Wasternack and Hause, 2002). In JA biosynthesis the AOC catalyses a crucial step by establishing the correct enantiomeric structure of jasmonates. Here, we identified four genes coding for AOCs of *A. thaliana*. All of them carry a chloroplast target sequence which seems to be active as revealed by immunocytochemical analysis (Figure 3). The anti-AOC2 antibody used for these experiments recognized all recombinant AOCs of *A. thaliana* (Figure 6C). Therefore, the lack of detectable AOC protein outside the chloroplast suggests that it is predominantly if not completely located in this organelle. In contrast to the existence of four different AOCs, only one gene coding for AOS exists in *A. thaliana* (Laudert *et al.*, 1996; Laudert and Weiler, 1998). The putative chloroplast target sequence detected for AOS (Laudert *et al.*, 1996) is shown here to be functionally active (Figure 7). Because of the short half-life of the AOS product in aqueous solution, a physical interaction of AOS and AOC might be assumed. However, we and others (E. Weiler, personal communication) failed to show such an interaction in a yeast two-hybrid system. It will be interesting to see whether the various AOCs differ in their location within sub-compartments of the chloroplast.

The existence of multiple genes coding for an enzyme of hormone biosynthesis is a common phenomenon. It has been reported for nitrilase in auxin biosynthesis (Bartel and Fink, 1994) and for 1-aminocyclopropane-1-carboxylate oxidase in ethylene biosynthesis (Barry *et al.*, 1996), and was found to be related to separate functions. Also for the AOC-catalysed step in JA biosynthesis, we expect non-redundant activity as suggested by preliminary data on specific expression of *AOC1*, *AOC2*, *AOC3* and *AOC4* in different organs. This is under study with promoter GUS lines of *AOC1*, *AOC2*, *AOC3* and *AOC4*. With probes specific for each *AOC*, a preferential AOC2 mRNA accumulation and a slightly higher AOC1 mRNA accumulation in the systemic response upon wounding of rosette leaves were found (Figure 5A, B), suggesting distinct roles in the wound response. Because of the systemic induction of AOS upon local wounding (Laudert and Weiler, 1998; Kubigsteltig *et al.*, 1999), it will be interesting to see whether AOS functions specifically with AOC1 in the systemic leaf. Another possibility for distinct functions of the different AOC genes is given by the occurrence of OPDA and dn-OPDA in *A. thaliana* (Figure 8) (Weber *et al.*, 1997; Reymond *et al.*, 2000). Possibly, the AOCs differ in their specificity to substrates originating from α-LeA and leading to OPDA or originating from hexadecatrienoic acid and leading to dn-OPDA. Also their pH optima might be different due to their different pIs (Table 1). However, such data are difficult to obtain, because the substrate for the AOC reaction is unstable, and to date only qualitative enzyme measurements are possible by using coupled enzyme assay.

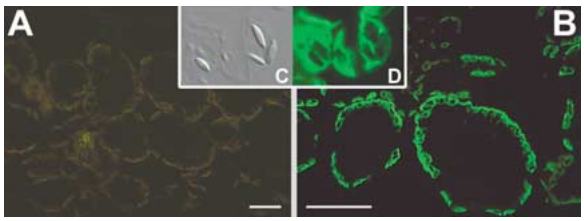

Figure 3. Immunocytological localization of AOC protein in rosette leaves of ecotype Columbia. Cross-sections of untreated leaves were probed with preimmune serum (A) or with anti-AOC-antibody raised against purified recombinant AOC2 of *A. thaliana* (B), followed by labelling with fluorescence-labelled secondary antibody. Whereas the labelling with pre-immune serum exhibits only the yellow-brown autofluorescence of chloroplasts (A), strong green fluorescence label within chloroplasts in B is indicative of the AOC protein. In C and D higher magnifications of B are shown to visualize starch granules by DIC image (C) or by the absence of fluorescence (D) (Bars: 50 μm in A and B).

JA biosynthesis is regulated by OPDA compartmentalization and a JA-mediated positive feedback loop

The stress-induced rise of jasmonates is a common phenomenon in all plants analysed so far. It is less understood, however, how this rise is regulated. A positive feedback was discussed based on data of transcriptional up-regulation of LOX, AOS, AOC and OPR3 upon treatment with jasmonates or biotic and

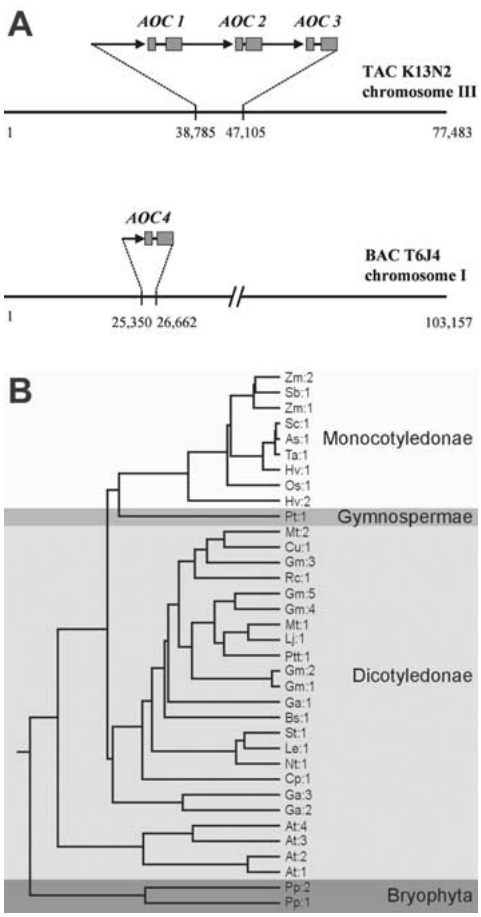

Figure 4. Mapping and genomic structure of *AOC1*, *AOC2*, *AOC3* and *AOC4* (A) and phylogenetic tree analysis (B). In A exons are denoted by closed rectangles and introns are represented by lines. *AOC1*, *AOC2* and *AOC3* map to TAC clone K 13N2 (chromosome 3, accession number AB028607) and *AOC4* maps to BAC clone T6J4 (chromosome 1, ACO11810). B shows a phylogenetic tree of 35 AOC proteins performed with the program Phylip 3.6. The AOC proteins refer to the following accession numbers in GenBank and the TIGR database (release of 7 February 2002): *Gossypium arboreum*, Ga:1 (TC926), Ga:2 (BG442722), Ga:3 (BG447327); *Medicago truncatula*, Mt:1 (AJ308489), Mt:2 (TC38027); *Citrus unshiu*, Cu:1 (C95219); *Ricinus communis*, Rc:1 (T15248); *Glycine max*, Gm:1 (TC79538), Gm:2 (TC79537), Gm:3 (TC79536), Gm:4 (TC84305), Gm:5 (TC85514); *Lotus japonicus*, Lj:1 (BI418833); *Populus tremula* and *Populus tremuloides*, Ptt:1 (BI128083 and BI069431); *Bruguiera sexangula*, Bs:1 (AB037929); *Solanum tuberosum*, St:1 (TC24555); *Lycopersicon esculentum*, Le:1 (AJ272026); *Nicotiana tabacum*, Nt:1 (AJ308487); *Citrus paradisi*, Cp:1 (BE205690); *Pinus taeda*, Pt:1 (BF609803 and BG275459); *Zea mays*, Zm:1 (TC92158), Zm:2 (TC92160); *Sorghum bicolor*, Sb:1 (TC25717); *Secale cerale*, Sc:1 (TC88); *Aegilops speltoides*, As:1 (BF291320); *Triticum aestivum*, Ta:1 (TC15431); *Hordeum vulgare*, Hv:1 (AJ308488), Hv:2 (BF256000); *Oryza sativa*, Os:1 (TC57764); *Arabidopsis thaliana*, At:1 (AJ308483), At:2 (AJ308484), At:3 (AJ308485), At:4 (AJ308486); *Physcomitrella patens*, Pp:1 (BJ182454 and AW509842), Pp:2 (BJ204710, BJ190940 and BJ168463).

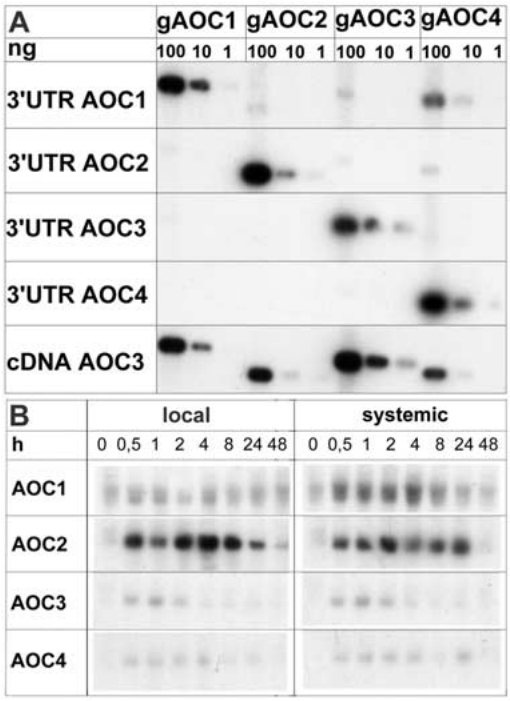

Figure 5. Expression analysis for *AOC1*, *AOC2, AOC3* and *AOC4* during local and systemic wound response. As shown in A, a 150 bp fragment of the 3′-untranslated region (3′-UTR) of each AOC hybridizes specifically with the corresponding genomic PCR fragment (100, 10, 1 ng) whereas the full-length cDNA of AOC3 hybridizes with genomic fragments of each AOC. In B the 150 bp 3′-UTR fragments were used to probe kinetics of local and systemic mRNA accumulation of AOC1, AOC2, AOC3 and AOC4 upon wounding.

abiotic stresses (Royo *et al.*, 1996; Laudert and Weiler, 1998; Howe *et al.*, 2000; Maucher *et al.*, 2000; Müssig *et al.*, 2000; Reymond *et al.*, 2000; Sivasankar *et al.*, 2000; Ziegler *et al.*, 2000). However, the following observations do not fit with a positive feedback in transcriptional regulation, at least in short-term treatments. First, despite transcriptional up-regulation of JA biosynthetic enzymes, there was no endogenous formation of jasmonates in barley or tomato leaves over 24 h (Kramell *et al.*, 2000; Miersch and Wasternack, 2000). Second, although AOS mRNA, protein and activity increased upon JA treatment in *Arabidopsis* (Laudert and Weiler, 1998), there was no corresponding OPDA accumulation (Stelmach *et al.*, 1999). Third, wounding or insect feeding led to a transient burst of JA within the first hour preceding the accumulation of AOS mRNA and AOC mRNA, respectively (Ziegler *et al.*, 2001; Stenzel *et al*., 2002).

Here, we detected basal occurrence of LOX, AOS and AOC proteins in all tissues of fully developed, un-

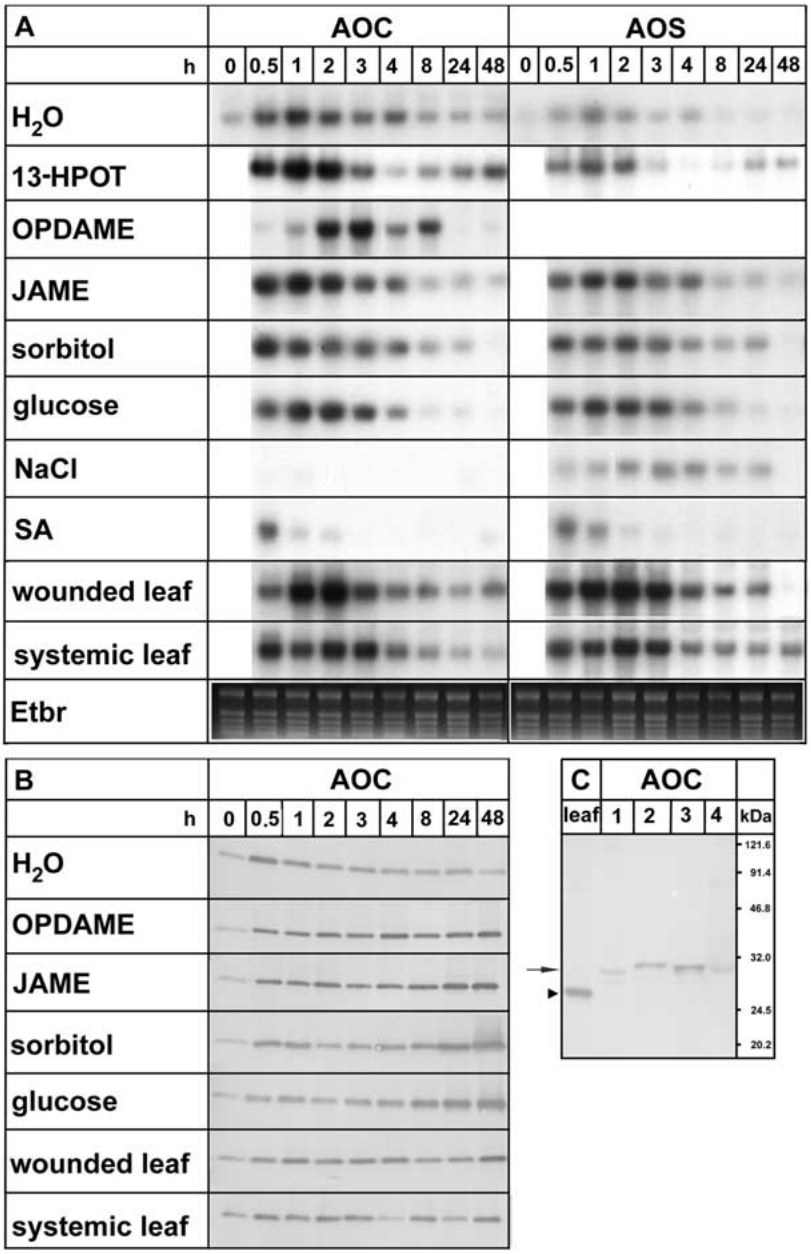

Figure 6. Expression of AOC genes in rosette leaves of *A. thaliana* in response to JAME and various other substance applications or stress conditions probed by northern blot analysis (A) and immunoblot analysis (B). For northern blot analysis rosettes were floated on distilled water, 50 μM 13-HPOT, 50 μM JAME, 1 M sorbitol, 0.5 μM glucose, 1 M NaCl or 50 μM SA for indicated times or wounded by crushing with a tailor's wheel in 3 mm distance. Per lane 20 μg of total RNA was loaded, and ethidium bromide staining served as loading control, as shown. All blots were hybridized with a ^{32}P-labelled full-length cDNA of AOC2. For immunoblot analysis (B), identical samples as in (A) were used for total protein extraction. Analysis was performed with 5 μg proteins per lane and an anti-AOC antibody diluted 1:5000. In (C) cross-reactivity is shown of the anti-AOC antibody raised against AOC2, with recombinant AOC1, AOC2, AOC3 and AOC4, indicated by an arrow as well as with AOC of a total leaf extract indicated by triangle. As a control 5 μg leaf protein was loaded. To detect cross-reactivity of the anti-AOC2 antibody with the other recombinant AOCs, the following amounts (ng per lane) was loaded: AOC1, 62.5; AOC2, 2.5; AOC3, 6.25; AOC4, 37.5.

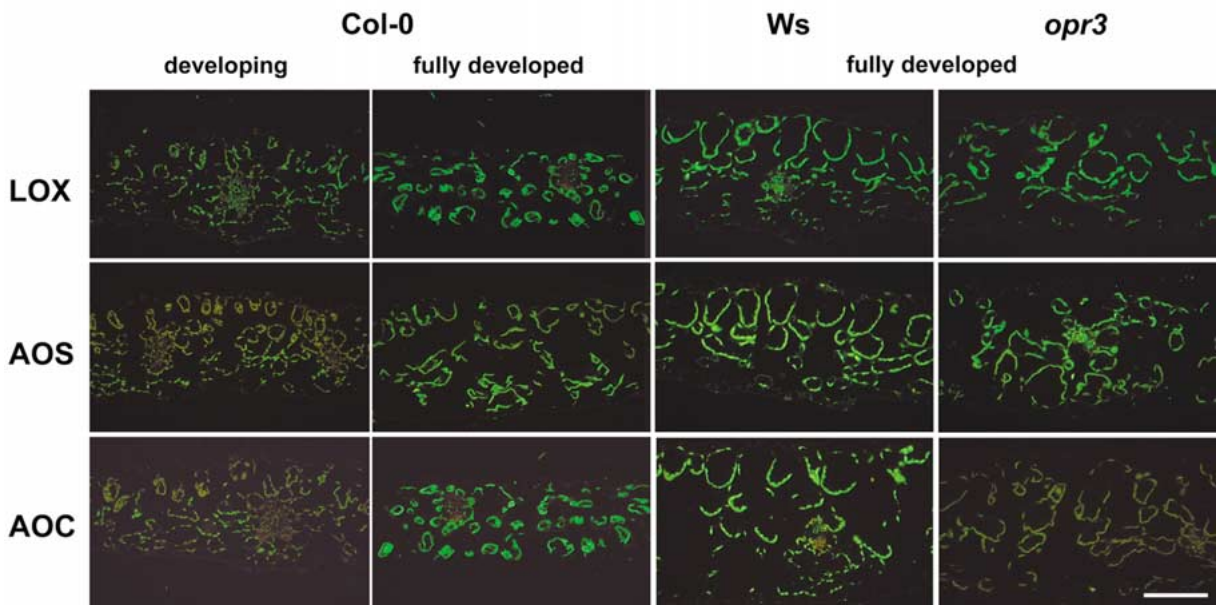

Figure 7. Immunohistochemical analysis on the occurrence of LOX, AOS, and AOC in untreated rosette leaves of the ecotypes Columbia (Col-O) and Wassilewskija (WS) and of the *opr3* mutant. Cross-sections were probed with an anti-LOX-antibody (dilution 1:500), an anti-AOS antibody (dilution 1:2000) or an anti-AOC antibody, used for Columbia in a dilution of 1:2000 and for Wassilewskija and *opr3* in a dilution 1:1000. The green fluorescence indicates occurrence of LOX, AOS and AOC protein, respectively. The fluorescence intensity is clearly more pronounced in fully developed leaves than in developing leaves of the ecotype Columbia. Whereas in the ecotype Wassilewskija and the *opr3* mutant similar label intensity is indicated for LOX and AOS proteins, the AOC protein level is significantly less in the mutant leaves than in the wild type. Yellow-brown autofluorescence appeared only upon treatment with the corresponding preimmune sera (data not shown, see also Figure 3A). Bars represent 100 μm in all figures.

treated rosette leaves (Figure 7). In contrast, tomato leaves exhibit specific expression of AOC in vascular bundles (Hause *et al.*, 2000a), accompanied by preferential accumulation of jasmonates in main veins compared to intercostal regions (Stenzel *et al.*, 2002). The constitutive high level of LOX, AOS and AOC in all leaf tissues of *A. thaliana* suggests that OPDA can be formed constitutively. Indeed, levels of free OPDA were detected of about 1 nmol per gram f.w. in the ecotype Columbia (Table 2) (Reymond *et al.*, 2000) and about 2.9 nmol/g in the ecotype Wassilewskija (Figure 8A). Furthermore, besides this remarkable amount of free OPDA, the main portion is esterified in chloroplast lipids (Stelmach *et al.*, 2001). Although its formation is unclear to date, one possibility is the action of LOX, AOS and AOC with esterified substrates, generating esterified OPDA. Upon wounding esterified OPDA is rapidly released (Stelmach *et al.*, 2001).

The remarkable level of free OPDA in untreated leaves (Figure 8) and the significant increase in LOX, AOS and AOC protein between young leaves to fully developed leaves (Figure 7) suggest a positive feed back loop during leaf development. *Arabidopsis* leaves treated with OPDA accumulate mRNA of LOX and AOS (Laudert and Weiler, 1998; Stintzi *et al.*, 2001) and of AOC (this paper). However, despite the remarkable levels of free OPDA (Figure 8) and esterified OPDA (Stelmach *et al.*, 2001) in untreated wild-type leaves, AOS RNA accumulated only very weakly and expression of AOC genes appeared only upon wounding (Figure 8C). Also in untreated *opr3* mutant leaves, AOC mRNA was not detectable despite high levels of OPDA. We suggest that over extended periods such as development of a wild-type leaf, release of OPDA from the chloroplast and generation of JA can occur at low levels. This amount of JA may induce expression of undetectable levels of mRNAs of LOX, AOS and AOC while leading to accumulation of the corresponding proteins detected by immunoblot and immunocytochemical analyses (Figures 7 and 8C). This scenario is supported by several findings. First, upon external application of JA or stress, both of which lead to an endogenous rise of jasmonates, mRNAs of LOX, AOS and AOC accumulate rapidly but transiently (Figure 6) (Bell and Mullet, 1993; Laud-

ert and Weiler, 1998). Second, the remarkable level of free and esterified OPDA might be biologically inactive in untreated developing leaves due to its confinement within the chloroplast. This is indicated by the fact, that OPDA-responsive *AOC* expression occurs in wounded but not unwounded *opr3* mutant leaves, and that wounded but not unwounded leaves of Thi2.1::*uidA* plants as well as VSP1::*uidA* plants show promoter activity (Bohlmann *et al.*, 1998; Ellis and Turner, 2001). In contrast, in untreated leaves of the *cet* (constitutive expression of thionin) mutant, which contains constitutively elevated JA levels, constitutive expression of the JA-responsive *Thi2.1* gene occurs (Hilpert *et al.*, 2001). Third, the proposed positive feed back loop seems to occur to a smaller extent in the JA-deficient OPDA-containing *opr3* mutant than in the wild type as shown by much less AOC protein in untreated mutant leaves (Figures 7 and 8C). Consequently, unwounded *opr3* mutant leaves contain less free OPDA than expected by the block in OPR3. This suggests that JA but not OPDA functions as the preferential signal of a putative feedback. Consistent with that is the significantly lower accumulation of AOS mRNA and AOC mRNA accumulation in wounded *opr3* leaves than in wild-type leaves. Interestingly, only the AOC protein but not the LOX and AOS proteins accumulated to less extent in *opr3* suggesting a preferential role of AOC in regulation of JA biosynthesis (Figures 7 and 8C). It will be interesting to see how OPR3 and β-oxidation steps, both assumed to be located within the peroxisomes, as well as a putative activation/transport of OPDA into peroxisomes attribute to overall regulation of JA biosynthesis. A positive feedback in JA biosynthesis was also suggested for senescing leaves which exhibited elevated JA levels and rise in mRNA accumulation of most genes coding for JA biosynthetic enzymes (He *et al.*, 2002).

13-HPOT, the initial substrate of the AOS branch was below the detection limit. However, JA levels are known to rise transiently upon wounding or other treatments generating α-LeA (Weber *et al.*, 1997; Bohlmann *et al.*, 1998; Reymond *et al.*, 2000). Consequently, application of 13-HPOT increases JA levels (data not shown) and induces expression of genes of AOC or other enzymes in JA biosynthesis (Figure 6) (Stenzel *et al.*, 2002). In contrast to leaves, in flowers of the mutant *dad1*, which is defective in a chloroplast-located PLA1, the JA levels were only 22% of that of the wild type suggesting limited generation of the initial substrate in JA biosynthesis (Ishiguro *et al.*, 2001).

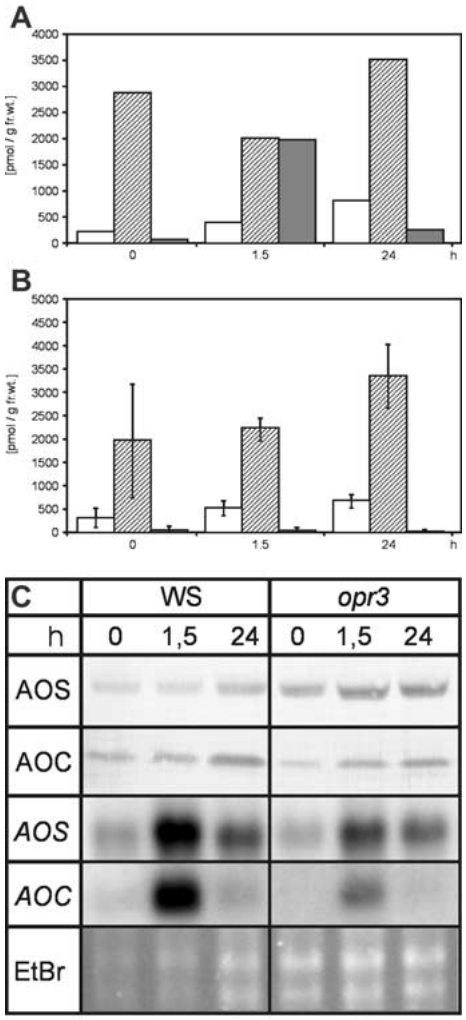

Figure 8. Levels of dn-OPDA, OPDA, and JA (A, B) and accumulation of mRNAs of AOS and AOC as well as of AOS protein and AOC protein (C) in unwounded and wounded rosette leaves of wild type (WS) and *opr3* mutant. For A and B, rosette leaves of 6-week old plants were wounded. For A leaves were pooled at least from four different plants of identical treatment and were subjected to analysis as described in Materials and methods. For B, three independent batches of leaf material were analysed for dn-OPDA (open bars), OPDA (hatched bars) and JA (filled bars). For C, an aliquot of leaf material as in A and B was subjected to northern blot and immunoblot analysis. Per lane 5 μg of total RNA was loaded, which was checked by ethidium bromide (EtBr) staining. For protein analysis 5 μg per lane was loaded and probed with anti-AOS antibody at a dilution of 1:2500 or with an anti-AOC antibody at a dilution of 1:5000.

To date the function of homologous proteins of DAD1 in rosette leaves is unknown. The undetectable amount of 13-HPOT and the high level of free and esterified α-LeA suggest the LOX reaction as another or additional control point in the substrate generation for JA biosynthesis. To date, however, there is no proof as to which of the six LOX forms found in the genome of *A. thaliana* functions in JA biosynthesis (Feussner and Wasternack, 2002). Four of them (LOX2, 3, 4, 6) are 13-LOXs and exhibit putative chloroplast target sequences. They might be detected in the immunocytochemical analysis shown in Figure 7, and they are candidates to function in JA biosynthesis possibly in different tissues of the plant. Based on previous data, it is very likely that in the case of leaves LOX2 is the major LOX involved in JA biosynthesis (Bell *et al.*, 1995).

The abundant occurrence of enzymes of JA biosynthesis within the chloroplast and rise in JA levels upon external stimuli such as wounding shown here, together with the recently found covalently linked OPDA derivatives, point to sequestration of enzymes and substrates as an important type of control in JA biosynthesis. LOX, AOS and AOC protein compartmentalized within the chloroplast (Figures 3 and 7) (Bell *et al.*, 1995; Laudert and Weiler, 1998) might function by substrate channelling during basal JA formation, and wounding may allow substrate accessibility by the relevant enzymes, thereby increasing JA formation. This model was discussed recently (Froehlich *et al.*, 2001) and is in accordance to previous data on 35S::LOX2*antisense* plants (Bell *et al.*, 1995), which exhibited a decrease in wound-induced JA formation only, but were still able to form residual levels of JA.

Another possibility in regulation of JA biosynthesis would be an activity control by post-translational modifications of the pre-existing enzymes such as AOC. Such a control would allow the plant to respond rapidly upon wounding or pathogen/insect attack by formation of JA which functions subsequently as a signal in defence gene expression. Such a rapid burst of JA and/or OPDA was found in tobacco and other plants upon insect attack (Heil *et al.*, 2001; Ziegler *et al.*, 2001) or wounding (Laudert *et al.*, 2000; Stenzel *et al.*, 2002, submitted). The functional role of subsequent transcriptional up-regulation of JA-biosynthetic enzymes observed here and by many others remains to be elucidated.

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