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Constitutive overexpression of allene oxide cyclase in tomato (*Lycopersicon esculentum* cv. Lukullus) elevates levels of some jasmonates and octadecanoids in flower organs but not in leaves

Otto Miersch[a], Heiko Weichert[b], Irene Stenzel[a], Bettina Hause[c], Helmut Maucher[d], Ivo Feussner[b], Claus Wasternack[a,*]

[a]*Institute of Plant Biochemistry, Department of Natural Product Biotechnology, Weinberg 3, D-06120 Halle/S, Germany*
[b]*Albrecht von Haller Institute for Plant Sciences, Department of Plant Biochemistry, Georg August University, Justus von Liebig Weg 11, D-370977 Göttingen, Germany*
[c]*Institute of Plant Biochemistry, Department of Secondary Metabolism, Weinberg 3, D-06120 Halle/S, Germany*
[d]*Institute for Plant Science and Crop Research, Department of Molecular Cell Biology, Corrensstr. 3, D-06466 Gatersleben, Germany*

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Abstract

The allene oxide cyclase (AOC), an enzyme in jasmonate biosynthesis, occurs in vascular bundles and ovules of tomato flowers which exhibit a tissue-specific oxylipin signature (Plant J. 24, 113-126, 2000). Constitutive overexpression of the *AOC* did not led to altered levels of jasmonates in leaves, but these levels increased upon wounding or other stresses suggesting regulation of jasmonate biosynthesis by substrate availability (Plant J. 33, 577-589, 2003). Here, we show dramatic changes in levels of jasmonic acid (JA), of 12-oxo-phytodienoic acid (OPDA), their methyl esters (JAME, OPDAME), and of dinor-OPDA in most flower organs upon constitutive overexpression of *AOC*. Beside a dominant occurrence of OPDAME and JA in most flower organs, the ratio among the various compounds was altered differentially in the organs of transgenic flowers, e.g. OPDAME increased up to 53-fold in stamen, and JA increased about 51-fold in buds and 7.5-fold in sepals. The increase in jasmonates and octadecanoids was accompanied by decreased levels of free lipid hydro(per)oxy compounds. Except for 16:2, the *AOC* overexpression led to a significant increase in free but not esterified polyunsaturated fatty acids in all flower organs. The data suggest different regulation of JA biosynthesis in leaves and flowers of tomato.

Keywords: Allene oxide cyclase; Transgenic tomato plants; Oxylipin levels; Flower organs

1. Introduction

Plants generate various lipid-derived signals in response to biotic and abiotic stresses. These compounds may activate distinct genes thus increasing defense. Jasmonates, the free acid (JA), the methyl ester (JAME) and JA- amino acid conjugates are well-known signals of the wound- and pathogen signalling pathways (Wasternack and Hause 2002; Creelman and Rao, 2002). Furthermore, the JA precursor 12-oxophytodienoic acid (OPDA), an octadecanoid compound, was identified as a JA-independent signal in plant pathogen interactions (Stintzi et al., 2001).

Both groups of compounds are formed within the lipoxygenase (LOX) pathway originating from α-linolenic acid (α-LeA). The initial reaction is the insertion of

Abbreviations: AOC, allene oxide cyclase; AOS, allene oxide synthase; 13-HPOT, (13*S*)-hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecadienoic acid; 13-HPOD, (13*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid; 13-HOT, (13*S*)-hydroxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic acid; 13-HOD, (13*S*)-hydroxy-(9*Z*,11*E*)-octadecadienoic acid; JA, jasmonic acid; JAME, jasmonic acid methyl ester; 13-KOD, (13*S*)-keto-(9*Z*,11*E*,15*Z*)-octadecadienoic acid; 13-KOT, (13*S*)-keto-(9*Z*,*11E*,15*Z*)-octadecatrienoic acid; α-LeA, α-linolenic acid; LOX, lipoxygenase; OPDA, 12-oxo-phytodienoic acid; OPDAME, 12-oxo-phytodienoic acid methyl ester; OPR, 12-oxo-phytodienoic acid reductase; PIN, proteinase inhibitor.

* Corresponding author. Tel.: +49-345-5582-1210; fax: +49-345-5582-1219.
E-mail address: cwastern@ipb-halle.de (C. Wasternack).

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molecular oxygen at carbon atom 13 catalyzed by a 13-LOX leading to (13*S*)-hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic acid (13-HPOT) (Fig. 1), whereas a 9-LOX can form 9-hydroperoxides. 13-HPOT is the substrate for seven different branches of the LOX pathway (Feussner et al., 2001). In case of JA biosynthesis an allene oxide synthase (AOS) converts the 13-HPOT into an unstable allene oxide which can rapidly decay into ketols or can cyclize non-enzymatically to racemic OPDA. In presence of an allene oxide cyclase (AOC), the (9*S*,13*S*)-enantiomer of OPDA (*cis*-(+)-OPDA) is formed. Due to this enantiomeric specificity, the AOC-catalyzed step is of special importance. The naturally occurring (+)-7-*iso*-JA carries this enantiomeric structure at the cyclopentanone ring. *Cis*-(+)-OPDA is metabolized by a specific OPDA reductase (OPR3) and three cycles of β-oxidation.

Most of the genes encoding enzymes of JA biosynthesis have been cloned. In tomato, four 13-LOXs (Heitz et al., 1997), two 13-AOSs (Howe et al., 2000; Sivasankar et al., 2000), one 9-AOS (Itoh et al., 2002), one AOC (Ziegler et al., 2000) and three OPRs (Strassner et al., 2002) have been characterized. Immunocytological analysis and import studies revealed that the putative

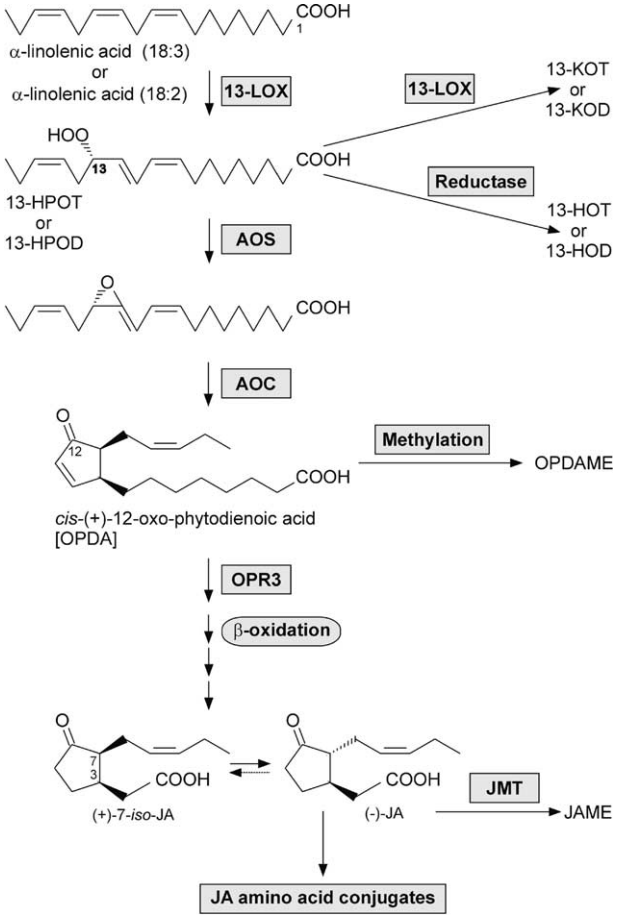

Fig. 1. Scheme on JA biosynthesis and some related branches of LOX pathway.

chloroplast transit peptides of 13-LOXs, 13-AOSs, and AOC are functional (Howe et al., 2000; Stenzel et al., 2003a; Ziegler et al., 2000). The OPR3 was localized in peroxisomes (Strassner et al., 2002).

Formation of JA is an essential step in a complex signalling cascade following wounding of tomato leaves as occurring by herbivore attack. Upon local wounding a prosystemin gene is expressed, and the 18-amino acid peptide systemin is processed from the pro-peptide prosystemin in the apoplast. Systemin can bind to the recently cloned membrane-located receptor which activates an intracellular signalling cascade including a MAPK, a Ca^{2+} ion flux, and a rise in α-LeA and JA levels (Conconi et al., 1996; O'Donnell et al., 1996; Stratmann and Ryan, 1997). Defense genes such as that coding for proteinase inhibitors (PINs) are finally activated thus attributing to an "immunization" of tomato plants against a new herbivore attack (Ryan, 2000).

The expression of genes encoding prosystemin and AOC occurs specifically in vascular bundles (Hause et al., 2000; Jacinto et al., 1997), and both of them are induced by JA and systemin, respectively. A preferential formation of JA in main veins of tomato leaves was found, and consequently, an amplification in wound signalling was proposed (Stenzel et al., 2003a). How the rise in JA upon wounding is regulated, is only partially understood. In tomato, most genes encoding enzymes of JA biosynthesis are JA-inducible (Strassner et al., 2002), and a feed forward regulation has been suggested. However, the rise in JA precedes the accumulation of corresponding mRNAs, and enzymes of JA biosynthesis occur constitutively in tomato leaves (Stenzel et al., 2003a). Furthermore, JA formation seems to be dependent on substrate availability, since in *AOC* overexpression lines of tomato elevation of JA in leaves occurs only upon wounding (Stenzel et al., 2003a). Similar substrate-dependency of JA formation was found with *AOS* overexpression lines of tobacco and *Arabidopsis* (Laudert et al., 2000).

In contrast to untreated wild type leaves, where only a residual level of JA and OPDA occurs (Stenzel et al., 2003a), the different flower organs contain a distinct oxylipin signature (Hause et al., 2000). This suggests that regulation of JA biosynthesis may differ between the leaf and the different flower organs. Another question in JA formation is its relationship to other branches of the LOX pathway. It has been previously shown that LOX products are shifted preferentially into the reductase branch upon salicylate treatment (Weichert et al., 1999), whereas preferential activity of the hydroperoxide lyase branch and the reductase branch were found upon jasmonate treatment (Bachmann et al., 2002). Consequently, a shift of metabolites between the different branches may occur, if an individual step of one branch is altered by constitutive overexpression or repression. Although such a transgenic approach is

artificial in respect to naturally occurring processes, the principal ability of metabolite levels, altered by the transgen, to affect reactions of the LOX pathway is unequivocally reflected. For the AOS branch constitutive overexpression of the AOC led to unchanged levels of jasmonates in leaves (Stenzel et al., 2003a). Additionally, except for 13-HOT and 13-HOD, levels of free and esterified lipid hydro(pero)xy compounds were only marginally affected in leaves of these transgenic plants (Weichert et al., 2003).

Here we addressed the question whether constitutive overexpression of the *AOC* alters levels of jasmonates and octadecanoids as well as of free and esterified lipid hydro(pero)xy compounds in flower organs. The organ-specific oxylipin signature was dramatically affected and levels of jasmonates and octadecanoids were elevated. This increase was accompanied with a diminished level of free lipid hydro(pero)xy compounds in most flower organs. The data indicate a remarkable shift in the 9-LOX and the 13-LOX activity in tomato flowers following *AOC* overexpression. In contrast to leaves, the substrate seems not to be limiting for JA biosynthesis in flower organs.

2. Results

2.1. Overexpression of AOC in tomato flowers

The specific expression of *AOC* in vascular bundles and ovules of tomato flowers is accompanied with an oxylipin signature differing among the flower organs (Hause et al., 2000). In order to inspect this signature in transgenic lines overexpressing or repressing *AOC*, we generated transgenic plants with the tomato AOC-cDNA *in sense* and *antisense* orientation under the control of the 35S promoter (Stenzel et al., 2003a). Inspection of the accumulation of AOC mRNA and AOC protein in leaves of five independent lines carrying the 35S*::AOCsense* construct revealed constitutive AOC expression in all lines with preferential abundance in S1 (T0) and S1.8 (T1) (Fig. 2a). All further analyses of the sense plants were done with line 1.8. In leaves of 9 independent lines carrying the 35S*::AOCantisense* construct no or only a residual amount of AOC transcript could be detected (Stenzel et al., 2003a). In order to check the AOC expression in transgenic flowers, we analyzed flowers of two months old plants (Fig. 3). As expected, the *AOC* overexpression led to accumulation of AOC protein in all flower tissues thereby contrasting from the specific occurrence of AOC in ovules and vascular bundles of wild type flowers (Fig. 3C versus A). Using a pre-immune serum a cross-reactivity was detected only with the epidermal layer of the ovules (Fig. 3B). The *35S::AOCantisense* lines still exhibited remarkable amounts of AOC protein in ovules and vascular bundles of flowers (Fig. 3D), even the *AOC* is a single copy gene in tomato (Ziegler et al., 2000). This contrasts to the clear *antisense* effect in leaves of these lines (Stenzel et al., 2003a) and might be caused by lower activity of the 35S promoter in flowers. Due to this amount of AOC in flowers of *antisense* line1.8, we

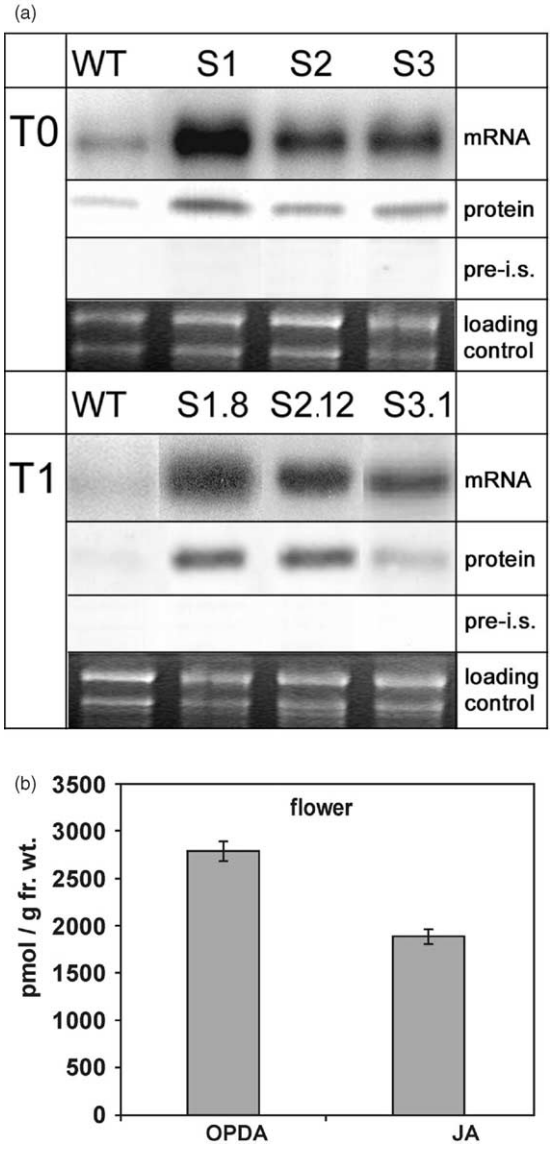

Fig. 2. Comparison in terms of AOC mRNA accumulation and AOC protein levels (a) in leaves of three out of five independent primary *35S::AOCsense* lines (TO) and T1 lines generated therefrom, as well as quantification of jasmonates and octadecanoids in wild type flower buds to show standards deviation (b). For (A) total RNA (20 μg per lane) and total protein extracts (10 μg per lane), were subjected to Northern blot analysis and immunoblot analysis, respectively. mRNA was probed with a ^{32}P-labeled full length *AOC* probe of tomato. Gel loading was checked by ethidium bromide staining. AOC protein was probed with an anti-AOC-antibody (dilution 1:5000) and a corresponding pre-immune serum (pre-i.s.) as described (Stenzel et al., 2003a). For (B) three different batches of about 50 flower buds pooled from 10 different plants, each of them two to three month old, were subjected to quantification of jasmonates and octadecanoids as described in Experimental.

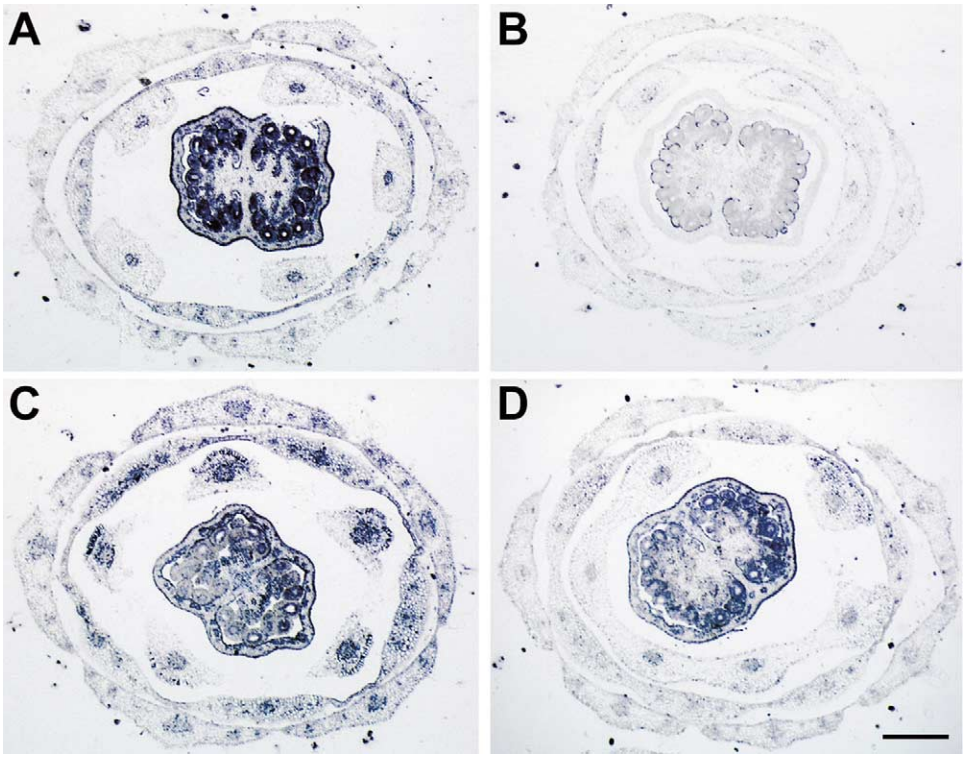

Fig. 3. Immunocytochemical localization of AOC in cross-sections of tomato flowers of 6-week old wild type (A), *35S::AOCsense* (C), and *35S::AOCantisense* (D) plants. Cross-sections (2 μm thickness) were probed with pre-immune serum (B) or the AOC protein was visualized by immunodecoration with rabbit anti-AOC antibody (A, C, D) both followed by a goat-anti-rabbit IgG antibody conjugated with alkaline phosphatase and staining as described in Experimental. With the anti-AOC-antibody in the wild type flowers strong label was found in vascular bundles of sepals, petals and stamens as well as in the ovules, whereas with the pre-immune serum only the epidermal layer of the ovules was labeled (B). In sense tissues the tissue-specific occurrence of AOC was compromised by constitutive expression in all tissues (C). In *antisense* tissues a significant label, most remarkably in ovules, indicated AOC protein. Bar: 500 μm for each picture.

analyzed only the overexpression lines compared to the wild type both in terms of jasmonates, octadecanoids, free and esterified lipid hydro(pero)xy compounds as well as free and esterified fatty acids.

2.2. Dramatic increase of OPDAME and JA as well as an altered oxylipin signature in flower organs by AOC overexpression

In comparing levels of jasmonates and octadecanoids in flowers of overexpression line 1.8 with that of wild type flowers numerous changes became obvious. To give an overview on the various changes, data are not given in numbers, but the absolute amounts in pmol per g f. w. were used for a graphical presentation in which the area of the circle and the area of the sector are indicative for the total amount and the amount of each compound, respectively (Fig. 4). The data can be summarized as follows:

1. Most of the compounds occurred at higher levels in flowers of the *sense* line indicating increased JA biosynthesis.
2. JA increased about 51-fold in buds and 7.5-fold in sepals following *AOC* overexpression. In contrast to the wild type, the JA level exceeded that of OPDA in most organs of transgenic flowers.
3. The dominant compound in all transgenic flower organs was the methyl ester of OPDA reaching 37 nmol per g f. w. in stamens of *sense* plants, which is a 53-fold increase compared to the wild type.
4. The ratio among the various jasmonates and octadecanoids, the oxylipin signature, was altered in the different flower organs following overexpression of *AOC*.
5. Low amount of JAME was found both in wild type and transgenic tissues.

2.3. Lipid hydro(pero)xy compounds occur preferentially esterified, but only the free forms exhibited decreased levels in flower organs of AOC overexpression lines

The dramatic increase of some octadecanoids and jasmonates in tomato flower organs following overexpression of *AOC* prompted us to inspect whether there was a simultaneous shift in levels of substrates used in JA biosynthesis as well as of compounds synthesized in related branches within the LOX pathway.

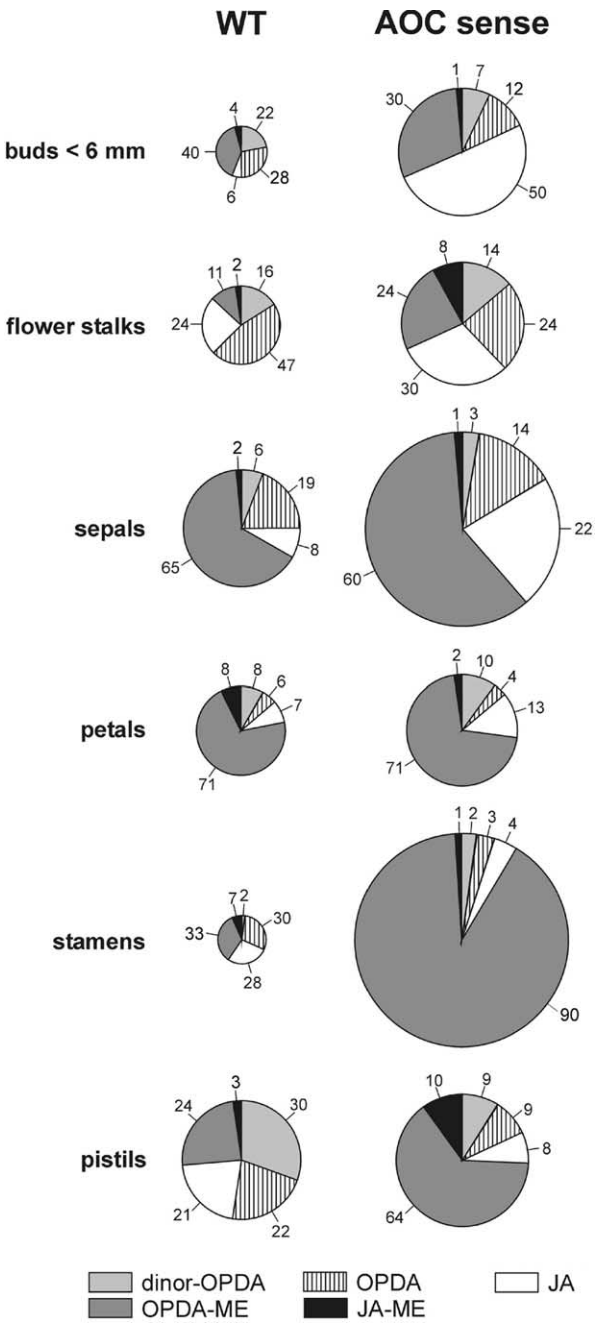

Fig. 4. Levels of jasmonate and octadecanoids in flower organs of wild type (*Lycopersicon esculentum* cv. Lukullus) plants and 35S::AOC*sense* plants (line 1.8, shown in Fig. 2). Tissues pooled from at least 65 flowers of at least 5 different two to three months old plants were extracted, purified by HPLC and quantified by GC-MS analysis as described in Experimental. The area reflects the total amount of compounds (wild type: buds, 2.4 pmol per g f. w.; flower stalks, 5.6 nmol per g f. w.; sepals, 12.6 nmol per g f. w.; petals, 7.2 nmol per g f. w.; stamens 2.1 nmol per g f. w.; pistils, 12.8 nmol g f. w.; *sense*: buds, 14.8 nmol per g. f. w; flower stalks, 13.8 nmol per g f. w.; sepals, 34.6 nmol per g f. w.; petals, 11.2 nmol per g f. w.; stamens 41.5 nmol per g f. w.; pistils, 16.0 nmol per g f. w.). The various sectors per circles are indicative for the amount of individual compounds (e. g. in *sense* stamens 1.5 nmol per g f. w. of JA, 0.29 nmol per g f. w. of JAME, 1.2 nmol per g f. w. of OPDA, 37.7 nmol per g f. w. of OPDAME and 0.9 nmol per g f. w. of dinor-OPDA). The amount of each compound is given in % of the total amount.

This metabolite profiling was done for free and esterified lipid hydro(pero)xy compounds (Fig. 5).

Most of these compounds were found preferentially in their esterified form in both the wild type and the 35S::*AOCsense* plants. In case of 13-HOD the esterified form accumulated up to a 20-fold excess over the free compound in most flower organs (Fig. 5c versus a). The amount of the esterified forms was not altered significantly by AOC overexpression except for 13-HOD, and the levels in the various organs did not differed dramatically for most compounds. An interesting characteristic, however, is the preponderance of esterified 13-HOD, and to a lesser extent of esterified 13-HOT, in most flower organs. This suggests a preferential activity of the 13-LOX pathway.

An overall comparison of wild type and transgenic flower organs revealed a significant decrease of levels in several free lipid hydro(pero)xy compounds. Free 9-HOD decreased most strongly from 19 to 3.2 nmoles per g f. w. in stamens and from 8.0 to 1.5 nmoles per g f. w. in pistils. These data suggest that *AOC* overexpression led to an increased consumption of free lipid hydro(pero)xy compounds including a decreased formation of 9-LOX products.

2.4. AOC overexpression leads to increased levels of free 18:1, 18:2 and 18:3 fatty acids but decreased levels of 16:2 in most flower organs

Due to the altered levels of free lipid hydro(pero)xy compounds in *AOC* overexpression lines the question arises whether the levels and composition of fatty acids were changed concomitantly. Inspection of the various flower organs in wild type and the 35S::*AOCsense* plants revealed for the latter an increase in the amount of free 18:1, 18:2 and 18:3 fatty acids in all flower organs (Fig. 6). This suggests that the elevated levels of jasmonates and octadecanoids observed in the overexpression line may induce release of these polyunsaturated fatty acid from their esterified form. In contrast, the level of free 16:2 decreased remarkably in most flower organs of the overexpression line. The total amount of each esterified fatty acid of flower organs differed not significantly between wild type and 35S::*AOCsense* plants. An interesting exception was again the 16:2 fatty acid. In pistils remarkable levels of free and esterified 16:2 fatty acids were detected in both the wild type and the transgenic plants.

3. Discussion

Jasmonates and octadecanoids are synthesized in response to environmental stresses and developmental cues. Flower development, tuberization and senescence are developmental processes exhibiting elevated JA

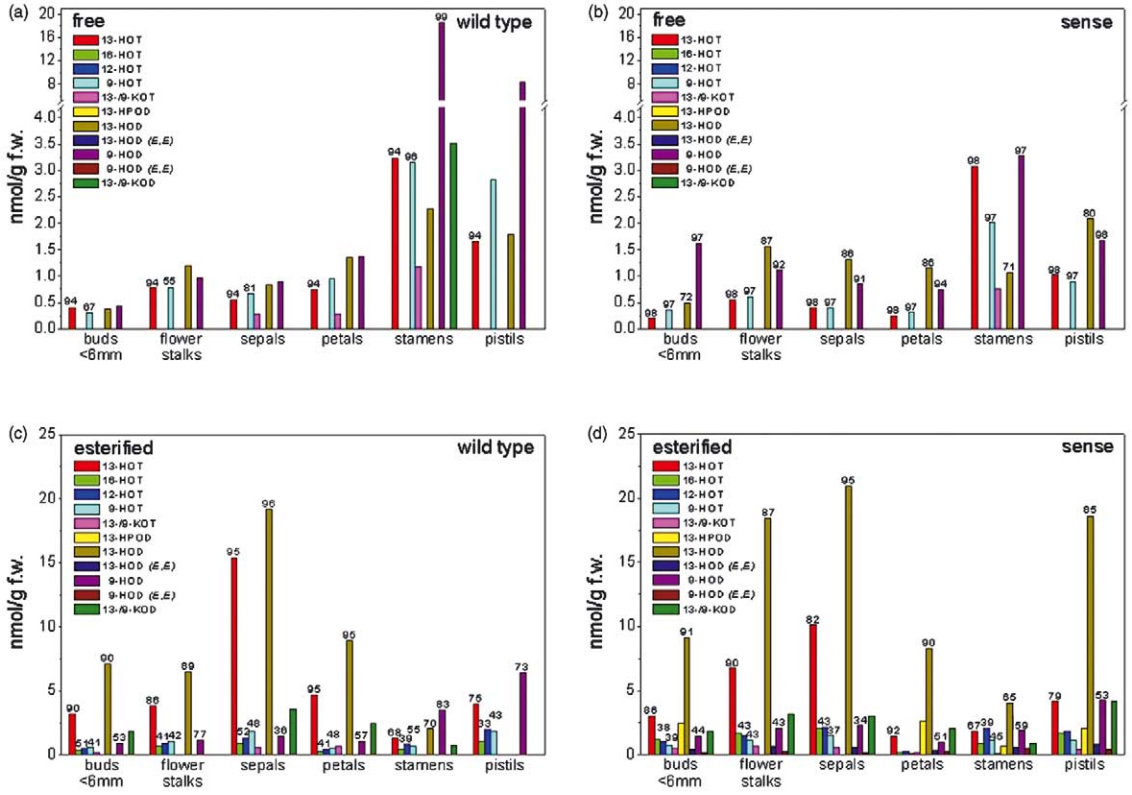

Fig. 5. Metabolic profiling of free (a, b) and esterified (c, d) lipid hydro(pero)xy compounds in flower buds and flower organs of wild type (a, c) and the *AOC* overexpression plants (b, d). The number per column gives the percentage of the *S*-enantiomer, indicating enzymatic synthesis.

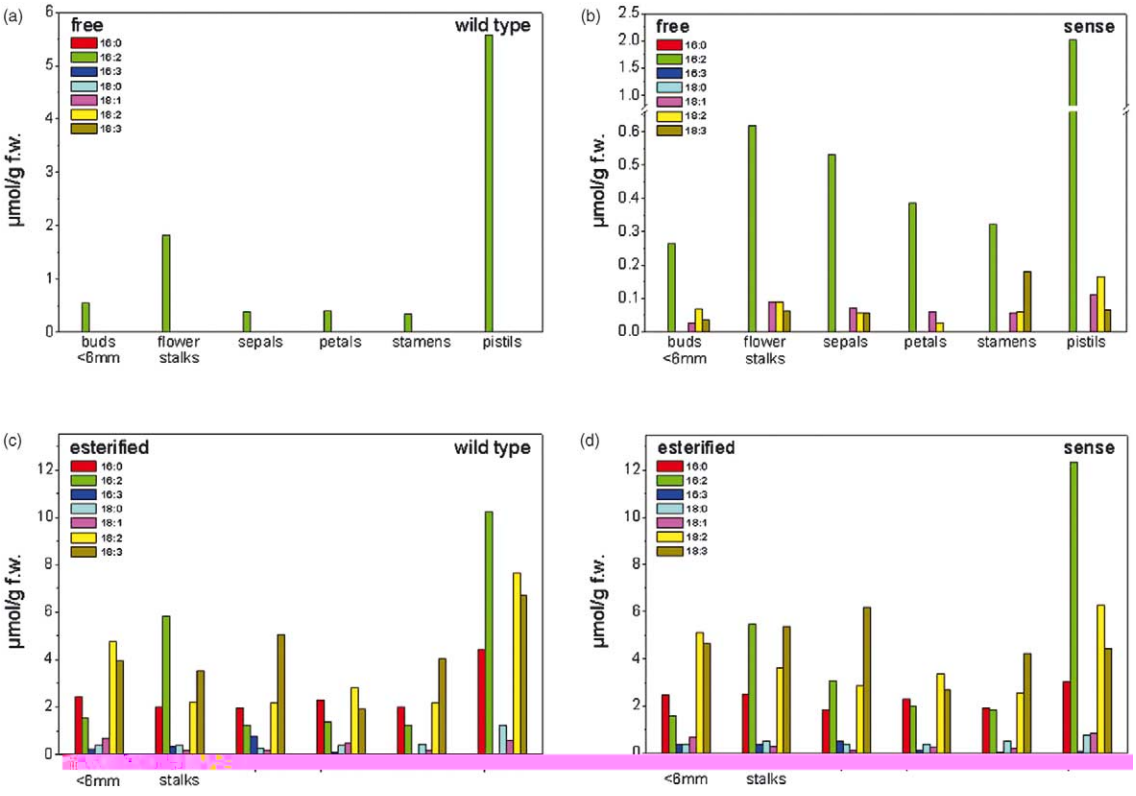

Fig. 6. Metabolic profiling of free (a, b) and esterified (c, d) fatty acids in flower buds and flower organs of wild type (a, c) and *AOC* overexpression plants (b, d).

levels or are JA dependent (Wasternack and Hause, 2002). In flower development of *Arabidopsis,* JA biosynthesis and perception are essential for male gametophyte development as indicated by the JA-insensitive mutant *coi1* (Feys et al., 1994) and the JA-deficient mutants *fad3-2fad7-2fad8*, *dde1*, *dad1* and *opr3* (Ishiguro et al., 2001; McConn and Browse, 1996; Sanders et al., 2000; Stintzi and Browse, 2000). In contrast, in tomato the JA insensitive mutant *jai-1* is female sterile (Li et al., 2001). Therefore, question arises how such species-specific differences in respect to JA perception and/or signalling evolved. Interestingly, in the different tomato flower organs JA accumulation differs remarkably. There is a preferential occurrence of OPDA in pistils, and of JA in flower stalks, whereas the ratio of various jasmonates and octadecanoids, the oxylipin signature, differed among the flower organs (Hause et al., 2000), leading to the question whether there are specific functions of jasmonates in the different flower organs. Here, we addressed the question, how such an oxylipin signature of flower organs and of leaves is altered upon overexpression of *AOC*, and whether an *AOC* overexpression has consequences for the amount and ratio of LOX pathway intermediates.

Overexpression or repression of genes encoding enzymes of hormone biosynthesis was repeatedly used to modulate hormone levels (Hedden and Phillips, 2000). In case of JA biosynthesis, constitutive overexpression of AOS in tobacco (Laudert et al., 2000; Wang et al., 1999), and in *Arabidopsis* (Park et al., 2001; Sanders et al., 2000) as well as of AOC in tomato (Stenzel et al., 2003a) increased levels of JA and OPDA in leaves only upon wounding or other external stimuli indicating that substrate availability regulates JA formation. In leaves this corresponds to the levels of preceding compounds of JA biosynthesis and related LOX products, the free and esterified lipid hydro(pero)xy compounds. They did not alter dramatically except for 13-HOT and 13-HOD (Weichert et al., 2003).

In contrast to these data, in flowers of the overexpression line numerous changes in the amount and composition of fatty acids, lipid hydro(pero)xy compounds, octadecanoids and jasmonates appeared: An interesting aspect described here for the first time, is the dominant occurrence of free 16:2 fatty acid in all flower organs compared to other free fatty acids which were under the detection limit in wild type plants. The 16:2 fatty acid was tentatively identified by its retention time in GC analysis as the (7*Z*, 10*Z*) form. In tomato leaves, 16:2 fatty acid could not be detected (Weichert et al., 2003) as in canola leaves (Madey et al., 2002). Its dominant occurrence in all flower organs was decreased by *AOC* overexpression. It will be interesting to see whether this preferential accumulation is accompanied with the formation of 16:2 derived oxylipins which have not been described so far.

The most intriguing results on levels of jasmonates, octadecanoids and lipid hydro(pero)xy compounds are (i) the difference in the amount between leaves and flowers, (ii) the specific ratio of these compounds in the various flower organs and (iii) the shift among these compounds by constitutive overexpression of *AOC*.

To (i): Most lipid hydro(pero)xy compounds reached in flower organs (Fig. 5) much higher levels than in leaves (Weichert et al., 2003) with preferential occurrence of the esterified form. In case of 13-HOT and 13-HOD levels in sepals, the esterified forms exceeded that of the free forms up to 20-fold. The preferential occurrence of 13-HOT and 13-HOD suggests that the 13-LOX branch is more active. Since the analytical techniques could be improved, increasing number of data can be expected on abundantly appearing esterified lipid-derived compounds as has been done for senescing flowers (Leverentz et al., 2002) and *Arabidopsis* leaves (Stenzel et al., 2003b). The 13-LOX-derived product OPDA was detected esterified in unwounded rosette leaves of *A. thaliana* 2-fold higher levels than the free form (Stelmach et al., 2001), and colneleic acid, the 9-LOX-derived product of the divinyl ether synthase reaction, was found to be a constituent of phospholipids in potato (Fauconnier et al., 2003).

Also jasmonates and octadecanoids reached much higher levels in flowers (Fig. 4) (Hause et al., 2000) than in untreated leaves (Stenzel et al., 2003a). So far there are no indications, and due to extractions procedures used it is highly unlikely, that JA is stored in esterified lipids of untreated leaves thereby attributing to much lower level than in flowers. There are clear indications for separate signalling properties for JA and OPDA (Alméras et al., 2003; Stintzi et al., 2001). Therfore it is interesting to see whether the specific amount of each compound for the various flower organs is of significance for a specific gene expression pattern. This specific amount reached remarkably high levels in case of OPDAME indicating preferential methylation of OPDA instead of ß-oxidation in sepals and petals, and also the dinor-OPDA levels were high in pistils. In this respect, it is interesting to note, that *cis*-regulatory sequences involved in the JA-inducible *PIN2* expression of flowers are different from those controlling expression in leaves (Lorberth et al., 1992).

To (ii): The different ratios of lipid hydro(pero)xy compounds in flower organs suggest that the tissues differ in the activity of the various LOX forms. An exceptional high level of free 9-HOD was found in stamens and pistils of wild type flowers (Fig. 5a). In leaves, 9-LOX products such as 9-HOD and 9-HOT increase with similar kinetics as PR gene expression in response to pathogen attack (Göbel et al., 2001, 2002). Thus, it is tempting to speculate that the organ-specific preference of 9-HOD represents a naturally occurring defense mechanism which protects these sink tissues against pathogen attack.

To (iii): The shift in levels of lipid hydro(pero)xy compounds following *AOC* overexpression indicates an altered regulation of the 9-LOX and the 13-LOX. In case of 9-HOD the dramatic decrease following AOC overexpression suggest a down-regulated 9-LOX branch (Fig. 5a), possibly caused by the dramatic increase in OPDAME and JA (Fig. 4) in these organs following *AOC* overexpression. The increase in the total amount of octadecanoids and jasmonates seems to be a consequence of *AOC* overexpression (Fig. 4) indicating that in contrast to leaves there is no substrate limitation in JA biosynthesis of flower organs. The characteristic levels of jasmonates and octadecanoids in the various flower organs shown for wild type flowers (Hause et al., 2000) were shifted remarkably by *AOC* overexpression. This suggests that the flower organs may regulate independently the formation of these compounds. Such organ-specific regulation in forming various signalling molecules might be of advantage at distinct stages of flower development and in the response to environmental factors. Elevation of JA and OPDA in distinct flower organs may induce genes coding for plant defense proteins or may function in plant communication. Numerous such genes are known and many of them are JA responsive such as proteinase inhibitors (Peña-Cortés et al., 1991), leucine amino peptidases (Tu et al., 2003), endo-β-1,4-glucanase and γ-thionins (Milligan and Gasser, 1995), and defensins (Lay et al., 2003). Among secondary compounds there are deterrant alkaloids known to be formed by enzymes located in the pistil (Lantin et al., 1999). In pistils several flower-specific terpene synthases attribute to emission of monoterpenes and sesquiterpenes which are discussed to be olfactory cues for pollinating insects (Chen et al., 2003). Another secondary metabolite, kaempferol, accumulates during pollination and wounding in stigma tissues which contain elevated levels of the JA-inducible PIN2 (Atkinson et al., 1993), of the AOC (Hause et al., 2003) and of JA (Fig. 4). In contrast to untreated leaves, where no shift in the compounds upon *AOC* overexpression occurred (Weichert et al., 2003; Stenzel et al., 2003a), the flower organ-specific alteration of octadecanoids, jasmonates and lipid hydro(pero)xy compounds following *AOC* overexpression will help to analyze putative biological consequences.

4. Experimental

4.1. Plant materials, growth and reagents

Lycopersicon esculentum Mill. cv. Lukullus and transgenic lines (Lukullus background) carrying the full length cDNA of the tomato AOC in *sense* or in *antisense* orientation under the control of the 35S promoter (Stenzel et al., 2003a) were grown as described (Wasternack et al., 1998). Flower buds (<6 mm) and sepals, petals, stamens, pistils and stalks were pooled from at least 65 different flowers of at least five wild type and five transgenic plants two to three months old. They were dissected with a razor blade and immediately frozen in liquid nitrogen. All jasmonates and octadecanoids were prepared and purchased, respectively, checked on purity and used as described (Hause et al., 2000; Maucher et al., 2000). The internal standards for GC-MS analysis [$^2H_6$]-JA and [$^2H_5$]-OPDA were prepared as described (Miersch, 1991; Zimmerman and Feng, 1978).

4.2. Extraction and quantitative analysis of jasmonates and octadecanoids

About 0.5 g f. w. in liquid nitrogen was homogenized in a mortar and extracted with 5 ml of 80% methanol (v/v). Appropriate amounts of [$^2H_6$]-JA and [$^2H_5$]-OPDA were added as internal standards for GC-MS analysis. Purification, fractionation and quantification was performed as described (Stenzel et al., 2003a). The standard deviation in GC-MS analysis was less than 10%. Due to the biologically pooled material and the great number of different compounds one series of extraction and measurements is presented. To show that this strategy is sufficient for the question under study, in one set of experiments standard deviations for the most important compounds such as JA, JAME, OPDA and OPDAME were calculated from three independent extraction procedures of biologically pooled material each of them measured in triplicates (Fig. 2b).

4.3. Separation and quantification of free and esterified fatty acids and of free and esterified lipid hydro(pero)xy compounds

From frozen tissues homogenized in a mortar, the fatty acids and lipid hydro(pero)xy compounds both in the esterified and the free form, were extracted, derivatized and separated into the corresponding positional and stereoisomeric forms as described (Stenzel et al., 2003b). Quantification was performed by HPLC (lipid hydro(pero)xy compounds) and GC (fatty acids) as described (Stenzel et al., 2003b). The standard deviation of analyses was less than 15%. Due to the biologically pooled material one series of measurements is presented.

4.4. Immunocytochemistry

Immunocytochemical analysis was performed as described (Hause et al., 2000) using cross-section (2 μm thickness) of PEG-embedded flower buds of two months old plants. The anti-AOC antibody raised against recombinant tomato AOC (Ziegler et al., 2000) was used in a section (2 μm thickness) of PEG-embedded

flower buds of two months old plants. The anti-AOC antibody raised against recombinant tomato AOC (Ziegler et al., 2000) was used in a dilution of 1:2000. As secondary antibody, anti-rabbit IgG conjugated with alkaline phosphatase (Chemicon Int., Hofheim, Germany) was used, according to the manufacturer's instructions. Staining was performed with nitrotetrazolium blue chloride (NBT) and 5-bromo-4-chloro-3-indanoyl phosphate (BCIP). Sections were analyzed by bright field microscopy using a Zeiss "Axioskop" (Zeiss, Jena; Germany). Pictures were taken with a CCD camera (Sony, Tokyo, Japan) and processed through the Photoshop 4.0 program (Adobe, Seattle; USA).

4.5. Extraction of RNA and proteins, Northern blot analysis and immuno blot analysis

Total RNA and proteins were extracted and subjected to Northern blot analysis and immuno blot analysis, respectively, as described (Stenzel et al., 2003a).

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References

Alméras, E., Stolz, S., Vollenweider, S., Reymond, P., Mène-Saffrané, L., Farmer, E.E., 2003. Reactive electrophile species activate defense gene expression in *Arabidopsis*. Plant J. 34, 205–216.

Atkinson, A.H., Heath, R.L., Simpson, R.J., Clarke, A.E., Anderson, M.A., 1993. Proteinase inhibitors in *Nicotiana alata* stigmas are derived from a precursor protein which is processed into five homologous inhibitors. Plant Cell 5, 203–213.

Bachmann, A., Hause, B., Maucher, H., Garbe, E., Vörös, K., Weichert, H., Wasternak, C., Feussner, I., 2002. Jasmonate-induced lipid peroxidation in barley leaves initiated by distinct 13-LOX forms of the chloroplast. Biol. Chem. 383, 1645–1657.

Chen, F., Tholl, D., D'Auria, J.C., Farooq, A., Pichersky, E., Gershenzon, J., 2003. Biosynthesis and emission of terpenoid volatiles from *Arabidopsis* flowers. Plant Cell 15, 481–494.

Conconi, A., Miquel, M., Browse, J.A., Ryan, C.A., 1996. Intracellular levels of free linolenic and linoleic acids increase in tomato leaves in response to wounding. Plant Physiol. 111, 797–803.

Creelman, R.A., Rao, M.V., 2002. The oxylipin pathway in *Arabidopsis*. In: Somerville, C.R., Meyerowitz, E.M. (Eds.), The Arabidopsis Book. American Society of Plant Biologists, Rockville, MD, pp. 1–24.

Fauconnier, M.-L., Williams, T.D., Marlier, M., Welti, R., 2003. Potato tuber phospholipids contain colneleic acid in the 2-position. FEBS Lett. 538, 155–158.

Feussner, I., Kühn, H., Wasternack, C., 2001. Lipoxygenase dependent degradation of storage lipids. Trends Plant Sci. 6, 273–378.

Feys, J.F., Benedetti, C.E., Penfold, C.N., Turner, J.G., 1994. *Arabidopsis* mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. Plant Cell 6, 751–759.

Göbel, C., Feussner, I., Hamberg, M., Rosahl, S., 2002. Oxylipin profiling in pathogen-infected potato leaves. Biochim. Biophys. Acta 1584, 55–64.

Göbel, C., Feussner, I., Schmidt, A., Scheel, D., Sanchez-Serrano, J.J., Rosahl, S., 2001. Oxylipin profiling reveals the preferential stimulation of the 9-lipoxygenase pathway in elicitor-treated potato cells. J. Biol. Chem. 276, 6267–6273.

Hause, B., Stenzel, I., Miersch, O., Wasternack, C., 2003. Occurrence of the allene oxide cyclase in different organs and tissues of *Arabidopsis thaliana*. Phytochem 64, 971–980.

Hause, B., Stenzel, I., Miersch, O., Maucher, H., Kramell, R., Ziegler, J., Wasternack, C., 2000. Tissue-specific oxylipin signature of tomato flowers: allene oxide cyclase is highly expressed in distinct flower organs and vascular bundles. Plant J. 24, 113–126.

Hedden, P., Phillips, A.L., 2000. Manipulation of hormone biosynthetic genes in transgenic plants. Curr. Opin. Biotechnol. 11, 130–137.

Heitz, T., Bergey, D.R., Ryan, C.A., 1997. A gene encoding a chloroplast-targeted lipoxygenase in tomato leaves is transiently induced by wounding, systemin, and methyl jasmonate. Plant Physiol. 114, 1085–1093.

Howe, G.A., Lee, G.I., Itoh, A., Li, L., DeRocher, A.E., 2000. Cytochrome P450-dependent metabolism of oxylipins in tomato. Cloning and expression of allene oxide synthase and fatty acid hydroperoxide lyase. Plant Physiol. 123, 711–724.

Ishiguro, S., Kawai-Oda, A., Ueda, J., Nishida, I., Okada, K., 2001. The defective in anther dehiscence 1 gene encodes a novel phospholipase A1 catalyzing the initial step of jasmonic acid biosynthesis, which synchronizes pollen maturation, anther dehiscence, and flower opening in *Arabidopsis*. Plant Cell 13, 2191–2209.

Itoh, A., Schilmiller, A., McCaig, B.C., Howe, G.A., 2002. Identification of a jasmonate-regulated allene oxide synthase that metabolizes 9-hydroperoxides of linoleic and linolenic acids. J. Biol. Chem. 277, 44658–46051.

Jacinto, T., McGurl, B., Francheschi, V., Delano-Freier, J., Ryan, C.A., 1997. Tomato prosystemin promoter confers wound-inducible, vascular bundle-specific expression of the b-glucuronidase gene in transgenic tomato plants. Planta 203, 406–412.

Lantin, S., O'Brien, M., Matton, D.P., 1999. Pollination, wounding and jasmonate treatments induce the expression of a developmentally regulated pistil dioxygenase at a distance, in the ovary, in the wild potato *Solanum chacoense*. Plant Mol. Biol. 41, 371–386.

Laudert, D., Schaller, A., Weiler, E.W., 2000. Transgenic *Nicotiana tabacum* and *Arabidopsis thaliana* plants overexpressing allene oxide synthase. Planta 211, 163–165.

Lay, F.T., Brugliera, F., Anderson, M.A., 2003. Isolation and properties of floral defensins from ornamental tobacco and petunia. Plant Physiol. 131, 1283–1293.

Leverentz, M.K., Wagstaff, C., Rogers, H.J., Stead, A.D., Chanasut, U., Silkowski, H., Thomas, B., Weichert, H., Feussner, I., Griffiths, G., 2002. Characterization of a novel lipoxygenase-independent senescence mechanism in *Alstroemeria* floral tissue. Plant Physiol. 130, 273–283.

Li, L., Li, C., Howe, G.A., 2001. Genetic analysis of wound signaling in tomato. Evidence for a dual role of jasmonic acid in defense and female fertility. Plant Physiol. 127, 1414–1417.

Lorberth, R., Dammann, C., Ebneth, M., Amati, S., Sánchez-Serrano, J.J., 1992. Promoter elements involved in environmental and developmental control of potato proteinase inhibitor II expression. Plant J. 2, 477–486.

Madey, E., Nowack, L.M., Thompson, J.E., 2002. Isolation and characterization of lipid in phloem sap of canola. Planta 214, 625–634.

Maucher, H., Hause, B., Feussner, I., Ziegler, J., Wasternack, C., 2000. Allene oxide synthases of barley (*Hordeum vulgare* cv. Salome)-tissue specific regulation in seedling development. Plant J. 21, 199–213.

McConn, M., Browse, J., 1996. The critical requirement for linolenic acid is pollen development, not photosynthesis, in an *Arabidopsis mutant*. Plant Cell 8, 403–416.

Miersch, O., 1991. Synthesis of (±)-(10-^{2}H,11-^{2}H$_3$,12-^{2}H$_3$)-jasmonic acid. Z. Naturforsch 46b, 1724–1729.

Milligan, S.B., Gasser, C.S., 1995. Nature and regulation of pistil-expressed genes in tomato. Plant Mol. Biol. 28, 691–711.

O'Donnell, P.J., Calvert, C., Atzorn, R., Wasternack, C., Leyser, H.M.O., Bowles, D.J., 1996. Ethylene as a signal mediating the wound response of tomato plants. Science 274, 1914–1917.

Park, J.M., Park, C.-J., Lee, S.-B., Ham, B.-K., Shin, R., Paek, K.-H., 2001. Overexpression of the tobacco *Tsi1* gene encoding an EREB/AP2-type transcription factor enhances resistance against pathogen attack and osmotic stress in tobacco. Plant Cell 13, 1035–1046.

Peña-Cortés, H., Willmitzer, L., Sánchez-Serrano, J.J., 1991. Abscisic acid mediates wound induction but not developmental-specific expression of the proteinase inhibitor II gene family. Plant Cell 3, 963–997.

Ryan, C.A., 2000. The systemin signaling pathway: differential activation of plant defensive genes. Biochim. Biophys. Acta 1477, 112–121.

Sanders, P.M., Lee, P.Y., Biesgen, C., Boone, J.D., Beals, T.P., Weiler, E.W., Goldberg, R.B., 2000. The *Arabidopsis DELAYED DEHISCENCE1* gene encodes an enzyme in the jasmonic acid synthesis pathway. Plant Cell 12, 1041–1061.

Sivasankar, S., Sheldrick, B., Rothstein, S.J., 2000. Expression of allene oxide synthase determines defense gene activation in tomato. Plant Physiol. 122, 1335–1342.

Stelmach, B.A., Müller, A., Hennig, P., Gebhardt, S., Schubert-Zsilavecz, M., Weiler, E.W., 2001. A novel class of oxylipins, *sn*1-*O*-(12-oxophytodienoyl)-*sn*2-*O*-(hexadecatrienoyl)-monogalactosyl diglyceride, from *Arabidopsis thaliana*. J. Biol. Chem. 276, 1282–1283.

Stenzel, I., Hause, B., Maucher, M., Pitzschke, A., Miersch, O., Ziegler, J., Ryan, C.A., Wasternack, C., 2003a. Allene oxide cyclase dependence of the wound response and vascular bundle specific generation of jasmonate in tomato—amplification in wound-signalling. Plant J. 33, 577–589.

Stenzel, I., Hause, B., Miersch, O., Kurz, T., Maucher, H., Weichert, H., Ziegler, J., Feussner, I., Wasternack, C., 2003b. Jasmonate biosynthesis and the allene oxide cyclase family of *Arabidopsis thaliana*. Plant Mol. Biol. 51, 895–911.

Stintzi, A., Browse, J., 2000. The Arabidopsis male-sterile mutant, *opr3*, lacks the 12-oxophytodienoic acid reductase required for jasmonate synthesis. Proc. Natl. Acad. Sci. U.S.A. 97, 10625–10630.

Stintzi, A., Weber, J., Reymond, P., Browse, J.A., Farmer, E.E., 2001. Plant defense in the absence of jasmonic acid: the role of cyclopentenones. Proc. Natl. Acad. Sci. U.S.A. 98, 12837–12842.

Strassner, J., Schaller, F., Frick, U.B., Howe, G.A., Weiler, E.W., Amrhein, N., Macheroux, P., Schaller, A., 2002. Characterization and cDNA-microarray expression analysis of 12-oxophytodienoate reductases reveals differential roles for octadecanoid biosynthesis in the local versus the systemic wound response. Plant J. 32, 585–601.

Stratmann, J.W., Ryan, C.A., 1997. Myelin basic protein kinase activity in tomato leaves is induced systemically by wounding and increases in response to systemin and oligosaccharide elicitors. Proc. Natl. Acad. Sci. U.S.A. 94, 11085–11089.

Tu, C.-J., Park, S.-Y., Walling, L.L., 2003. Isolation and characterization of the neutral leucine aminopeptidase (LapN) of tomato. Plant Physiol. 132, 243–255.

Wang, C., Avdiushko, S., Hildebrand, D.F., 1999. Overexpression of a cytoplasm-localized allene oxide synthase promotes the wound-induced accumulation of jasmonic acid in transgenic tobacco. Plant Mol. Biol. 40, 783–793.

Wasternack, C., Hause, B., 2002. Jasmonates and octadecanoids: Signals in plant stress responses and plant development. Progr. Nucleic Acid Res. Mol. Biol. 72, 165–221.

Wasternack, C., Ortel, B., Miersch, O., Kramell, R., Beale, M., Greulich, F., Feussner, I., Hause, B., Krumm, W., Boland, W., Parthier, B., 1998. Diversity in octadecanoid-induced gene expression of tomato. J. Plant Physiol. 152, 345–352.

Weichert, H., Stenzel, I., Berndt, E., Wasternack, C., Feussner, I., 1999. Metabolic profiling of oxylipins upon salicylate treatments in barley leaves- preferential induction of the reductase pathway by salicylate. FEBS Lett. 464, 133–137.

Weichert, H., Maucher, H., Hornung, E., Wasternack, C., Feussner, I., 2003. Shift in fatty acid and oxylipin pattern of tomato leaves following overexpression of the allene oxide cyclase. In: Murata, N. (Ed.), Advanced Research of Plant Lipids. Kluwer Academic Publishers, Japan, pp. 275–278.

Ziegler, J., Stenzel, I., Hause, B., Maucher, H., Miersch, O., Hamberg, M., Grimm, M., Ganal, M., Wasternack, C., 2000. Molecular cloning of allene oxide cyclase: The enzyme establishing the stereochemistry of octadecanoids and jasmonates. J. Biol. Chem. 275, 19132–19138.

Zimmerman, D.C., Feng, P., 1978. Characterization of a prostaglandin-like metabolite of linolenic acid produced by a flax seed extract. Lipids 13, 313–316.