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BIOTIC AND ABIOTIC STRESS

Wound response in passion fruit (*Passiflora* f. *edulis flavicarpa*) plants: gene characterization of a novel chloroplast-targeted allene oxide synthase up-regulated by mechanical injury and methyl jasmonate

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Abstract The induction of a chloroplast-localized 13-lipoxygenase (13-LOX) in passion fruit leaves in response to methyl jasmonate (MeJa) was previously reported. Since allene oxide synthase (AOS) is a key cytochrome P450 enzyme in the oxylipin pathway leading to AOS-derived jasmonates, the results above led in turn to an investigation of AOS in our model plant. Spectrophotometric assays showed that 24 h exposure of MeJa caused a high increase in 13-hydroperoxy linolenic acid (13-HPOT) metabolizing activity in leaf tissue. Western analysis using polyclonal antibodies against tomato AOS strongly indicate that, at least a part of the 13-HPOT metabolizing capacity can be attributed to AOS activity. We cloned the cDNA from a novel AOS encoding gene from passion fruit, named P*f*AOS. The 1,512 bp open reading frame of the AOS–cDNA codes a putative protein of 504 amino acid residues containing a chloroplast target sequence. Database comparisons of the deduced amino acid sequence showed highest similarity with dicot AOSs. Immunocytochemistry analysis showed the compartmentalization of AOS in chloroplasts of MeJa treated leaves, corroborating the predicted subcellular localization. Northern analysis showed that AOS gene expression is induced in leaf tissue in response to mechanical wounding and exposure to MeJa. In addition, such treatments caused an increase in papain inhibitor(s) in leaf tissue. Taken together, these results indicate that P*f*AOS may play an important role in systemic wound response against chewing insect attack. Furthermore, it can be useful as a tool for understanding the regulation of jasmonates biosynthesis in passion fruit.

Keywords Allene oxide synthase · Methyl jasmonate · Passion fruit · Wound response

Abbreviations

LOX	Lipoxygenase
AOS	Allene oxide synthase
13-HPOT	13-Hydroperoxy linolenic acid
MeJa	Methyl jasmonate
JA	Jasmonic acid
OPDA	12-Oxo-phytodienoic acid
CYP	Cytochrome P450 subfamily
HPL	Hydroperoxide lyase
ORF	Open reading frame

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UTR	Untranslated region
PVPP	Polyvinylpolypyrolidone
5′ RACE	5′ Rapid amplification of cDNA ends
BANA	*N*-Benzoyl-1-arginine-2-naphthylamide

Introduction

In plants, environmental stress and developmental cues generate diversity in the metabolites produced by the oxidative metabolism of polyunsaturated fatty acids, collectively called oxylipins (Wasternack and Hause 2002; Farmer et al. 2003). Therefore, these biologically active compounds can play various roles when plants cope with a variety of stimuli. Since the jasmonates can act as signal molecules, among oxylipins, they play a very important function in plant defense mechanisms against insect and pathogen attacks (Halitschke and Baldwin 2005; Pozo et al. 2005). The role of jasmonates (more precisely, jasmonic acid—JA and methyl jasmonate—MeJa) in plant defense against insect predation was initially proposed in the beginning of the 1990s by Ryan's group, when it was demonstrated the powerful effect of such molecules upon the induction of serine proteinase inhibitors in plants from *Solanaceae* and *Fabaceae* families (Farmer and Ryan 1990, 1992; Farmer et al. 1992). After these findings, several research groups are focused to understand better the role of jasmonates in the regulation of developmental and defense-related processes.

JA occurs in a variety of modified forms, including the methyl ester, glycosyl esters, and amide-linked conjugates with different aminoacids (Wasternack and Parthier 1997). Jasmonate dependent responses contribute significantly to the defense status of the plants by increasing the amount of several defensive proteins and/or phytochemicals that collectively abolish or retard the aggressor's invasion (Kessler and Baldwin 2002; Farmer et al. 2003; Zhao et al. 2005). Jasmonic acid is the end product of the octadecanoid pathway, synthesized via a series of reactions beginning with the release of linolenic acid and its conversion to 13-hydroperoxide by the action of 13-lipoxygenase (13-LOX). The generated molecules serve as important intermediates in branch points in this pathway. But only the dehydration of 13-HPOT by allene oxide synthase (AOS) generates the allene oxide intermediate for JA biosynthesis (Blée 2002; Schaller et al. 2005). Due to the instability of this intermediate it is either chemically hydrolyzed to α- and γ-ketol and racemic 12-oxo-phytodienoicacid (OPDA) or is cyclized by allene oxide cyclase to enantiomeric OPDA, which subsequently undergoes reduction and three cycles of β-oxidation to yield JA.

Due to its importance for JA biosynthesis, several full-length AOS-encoding cDNAs from dicot plants have been isolated and characterized, for instance, flax (Song et al. 1993), guayule (Pan et al. 1995), *Arabidopsis* (Laudert et al. 1996), tomato (Howe et al. 2000; Sivasankar et al. 2000; Itoh et al. 2002), tobacco (Ziegler et al. 2001), and in a lesser extent from monocot models, barley (Maucher et al. 2000, 2004), and rice (Agrawal et al. 2002; Haga and Iino 2004). Plant AOS are cytochrome P450 enzymes, most of them so far belong to a subfamily designated as CYP74A. Since they do not require molecular oxygen or reducing equivalents from NADPH for their activity, plant AOSs are described as non-classical P450s (Schaller et al. 2005). Treatments known to trigger the defense response in plants such as mechanical wounding, herbivory, caterpillar regurgitants, jasmonates, pathogens, and elicitors also lead to an increase of AOS transcripts and/or JA levels (Laudert and Weiler 1998; Howe et al. 2000; Sivasankar et al. 2000; Maucher et al. 2000; Ziegler et al. 2001; Agrawal et al. 2002; Mei et al. 2006). Such results strongly indicate the importance of AOS for the production of jasmonates and defense response mechanisms. Transgenic studies using *Arabidopsis* and tobacco plants also revealed the regulatory role of AOS in the biosynthesis of JA (Wang et al. 1999; Laudert et al. 2000; Park et al. 2002).

Brazil is among the largest producers of passion fruit (Matsuura and Folegatti 2004; Pires and Mata 2004) due to its large use in commercial juice production, making this crop an interesting model to study plant defense mechanisms. Our previous data suggested the importance of the octadecanoid pathway for the wound response in passion fruit plants (Rangel et al. 2002). In this work, we focus our investigation on signal transduction pathway leading to the induction of defensive mechanisms in passion fruit, particularly on a key enzyme of JA biosynthesis. The results described here further support the importance of jasmonates for the induction of defensive responses when passion fruit plants are attacked by herbivorous insects and possibly by pathogens.

Materials and methods

Plant material and treatments

Passion fruit (*Passiflora edulis* f. *flavicarpa*) were purchased from local market of Campos dos Goytacazes, a city located in the northern region of Rio de Janeiro State, Brazil. Seeds were collected, dried at room temperature and stored at 4°C in the dark until used. Plants were grown in peat pots and maintained in environmental chambers for 17 h under 300 mE m^{-2} s^{-1} light at 28°C and for 7 h in the dark at 18°C. Three-week-old plants having two developed leaves and a small apical leaf were used in all the experiments. Lower leaves were wounded multiple times (3–4)

with a hemostat, a total wounded area of ~32 mm^2 per leaf. Developed upper leaves were assayed as systemic ones. Intact plants were exposed continuously to MeJa vapors in closed Plexiglas boxes as described by Farmer and Ryan (1990). Briefly, 2 μL of absolute MeJa (Bedoukian Research) was applied on a cotton wick inside the 1 L box. All plants were maintained in environmental chambers under constant light until assay. For all the experiments, period of exposure to treatments are given in figure legends.

Protein extraction, gel electrophoresis and immuno-blot analysis

The passion fruit leaves were ground to a fine powder in liquid N_2, 10% (w/w) insoluble polyvinylpolypyrolidone (PVPP) was added to it and total protein was extracted by addition of 3 mL ice-cold extraction buffer (50 mM potassium phosphate, pH 7.0) to 1 gram of powdered tissue. The homogenate was centrifuged at 10,000*g* for 30 min at 4°C. Otherwise stated, the recovered crude supernatant was used for the following experiments. Protein concentration was determined using the Bradford assay kit and bovine serum albumin as standard protein according to the manufacturer's instructions (BioRad). Proteins were analyzed in 10% (w/v) SDS polyacrylamide gel according to Laemmli (1970). The transfer of proteins to nitrocellulose membranes was carried out as described by Towbin et al (1979). Membranes were probed with pre-immune serum or immune serum against tomato AOS (gift from Dr. Gregg A. Howe, Michigan State University, USA). Blots were developed using chemiluminescent detection (Amersham ECL reagent) according to the manufacturer's instructions. Membranes probed with immune serum against tomato cystatin were developed using Protein A-peroxidase (Sigma) and DAB (3,3′-diaminobenzidine, BioRad) following the manufacturer's instructions.

Preparation of fatty acid hydroperoxides and spectrophotometric assays

13-Hydroperoxides of linolenic acid were prepared as described by Vick (1991), using a partially purified MeJa inducible 13-LOX from passion fruit leaves (Rangel et al. 2002). Linolenic acid was purchased from Sigma. The 13-HPOT metabolizing activity in leaf crude extracts was measured spectrophotometrically by monitoring the rate of decrease in absorbance at 234 nm that resulted from disruption of the conjugated diene bond in the substrate (Zimmerman and Vick 1970). Briefly, enzymatic assays were performed at 25°C in 1 mL of 50 mM potassium phosphate (pH 7.0) containing an amount of 13-HPOT equivalent to an absorbance of 0.4 at 234 nm. The assay was started by addition of 70 μg of total protein. Assays performed without substrate showed no decrease in absorbance (data not shown).

Cloning of the full-length P*f*AOS–cDNA

First strand cDNA was synthesized in a 50 μL reaction mixture with a Superscript RT–PCR Kit (Invitrogen) according to the manufacturer's protocol using 5 μg of total RNA isolated from leaves of passion fruit plants treated with MeJa for 12 h. Initially, the cDNA central region was amplified by PCR from the first strand cDNA prepared above, using two degenerate primers designed based on consensus sequences from published plant AOSs. Forward and reverse primers used for PCR were 5′-TTCAC(CT)GG (AT)ACTT(AT)C(AG)TGCC-3′ and 5′-TCCGG(CT)CC (AG)TT(AC)GACCA(CT)-3′, respectively. The resulting amplified fragment was cloned into a TOPO vector (Invitrogen) and both strands sequenced using a dye-terminator cycle sequencing reaction kit (Perkin Elmer Applied Biosystems) and an automated DNA sequencer (Genetic Analyzer ABI 370, Perkin Elmer Applied Biosystems). 5′-Rapid amplification of cDNA ends procedure was used (5′ RACE kit, Invitrogen), following the manufacturer's protocol to obtain the remaining sequence from the 5′ end of P*f*AOS–cDNA. First strand cDNA was synthesized from the total RNA prepared from passion fruit leaves treated with MeJa for 12 h as a template. The sequence of the gene-specific primer used for this reaction was 5′-GCCTTTCTC TGCGATTTCCTTCTC-3′. Polymerase chain reaction amplification of the dC-tailed cDNA was carried out using the manufacturer's abridged anchor primer and a gene-specific primer having the sequence 5′-CCGTTAAA CAACTCGCTGAAAC-3′. The PCR products were re-amplified using the manufacturer's abridged universal amplification primer and the nested gene-specific primer having the sequence 5′-CTCGGGGACTATATGGTCGCT GCG-3′. The final amplified cDNA fragment was cloned into TOPO vector (Invitrogen) and both strands were sequenced as described above. The overlapping region of the cDNA central region with the structure of the 5′-Untranslated region (5′-UTR) of P*f*AOS was confirmed, including the presence of the in-frame stop codon 48 nucleotides upstream of the initiator *Met* codon. The 3′ end fragment of P*f*AOS–cDNA was amplified by RT–PCR. First strand cDNA was obtained using the Superscript RT–PCR Kit (Invitrogen) according to the manufacturer's protocol, using total RNA prepared from passion fruit leaves treated with MeJa for 12 h as a template. A cDNA fragment was amplified using a gene-specific primer

5′-GCCGCATCAGTATGGCAAAGC-3′ and oligo dT by PCR. The resulting cDNA fragment was cloned into TOPO vector (Invitrogen) and both strands were sequenced as described above. The overlapping region of 220 bp with the cDNA core fragment and the structure of 3′-UTR of P*f*AOS–cDNA were confirmed.

DNA sequencing analysis

The sequence data were analyzed using the BLAST program (http://www.ncbi.nlm.nih.org/Blast, Altschul et al. 1990). Nucleotide and amino acid sequence alignment was performed using CLUSTAL W program against the GenBank and EMBL DNA databases (http://www.genebee.msu.su/clustal/basic.htm) with default settings (Thompson et al. 1994). Alignment of the available full-length sequence of AOS–cDNA with P*f*AOS–cDNA was carried out using BioEdit program 7.0 (Hall 1999). The phylogenetic tree was constructed using Phylodendron Phylogenetic Tree Drawing (http://www.iubio.bio.indiana.edu/treepp/).

Northern and Southern blot analysis

Leaf tissue was ground to a fine powder in liquid N_2 and total RNA was extracted using Trizol reagent (Invitrogen) following the manufacturer's instructions. RNA samples (15 μg) were separated by electrophoresis through formaldehyde–1.4% agarose gel and blotted onto a nitrocellulose membrane (BioRad). The membranes were hybridized with ^{32}P-labelled P*f*AOS–cDNA core fragment. Probes were prepared using Mega prime DNA labeling system (Amersham) following the manufacturer's instructions. Hybridization was performed as described by Sambrook and Russell (2001). The hybridized membranes were washed two times with 0.1× SSC and 0.1% (w/v) SDS at 55°C for 30 min, and exposed to a X-ray film (Kodak) using one intensifying screen for 48 h at –80°C. Genomic DNA was extracted from leaf tissue using DNAzol (Invitrogen) following the manufacturer's instructions. DNA samples (20 μg) were digested with restriction enzymes (*Eco*RI, *Not*I and *Sma*I), fractioned through 0.8% agarose gels, transferred to nitrocellulose membrane (BioRad), and probed with ^{32}P-labelled P*f*AOS–cDNA central region that does not contain restriction site for these enzymes. Hybridizations and washing conditions were performed as describe above.

In vitro papain inhibitory assay

Leaf tissue was ground to a fine powder in liquid N_2, 10% (w/w) insoluble PVPP was added and total protein was extracted by adding 3 mL of ice-cold buffer (50 mM sodium phosphate, pH 6.0) to 1 g of powdered tissue. The homogenate was centrifuged at 4°C for 30 min at 10,000*g*. The supernatant was recovered as a crude extract. Measurements of the inhibition activity of crude leaf extract (70 μg) were performed using *N*-benzoyl-1-arginine-2-naphthylamide (BANA, Sigma), following the procedure described by Abe et al. (1994). In all experiments, 1 μg of papain was used. Inhibitory activity was detected as a reduction of BANA hydrolyzing activity of papain.

Immunocytochemistry analysis

Leaf samples were fixed in 50 mM cacodylate buffer (pH 7.4) containing 0.1% glutaraldehyde and 4% paraformaldehyde. After rinsing with 50 mM cacodylate buffer (pH 7.4), samples were dehydrated in graded methanol solutions and embedded in LR GOLD resin. Ultrathin sections (60 nm) were mounted on nickel grids and submitted to the immunocytochemistry assay as described: grids were bathed for 30 min with PBS buffer (10 mM NaH_2PO_4, 150 mM NaCl, pH 7.3) containing 1% (v/v) Tween-20 and 2 h with 50 mM NH_4Cl, pH 5.25. In order to block non-specific protein-binding sites, grids were incubated with PBS buffer + 1% BSA for 2 h. The grids were then incubated for 2 h with polyclonal antibodies against tomato AOS diluted (1:200) with PBS + 1% BSA. As control, sections were incubated with pre-immune serum under same conditions. After rinsing 8 × 5 min in PBS + 1% BSA and 4 × 5 min in PBS alone, sections were floated for 2 h on a solution containing anti-rabbit IgG-gold (10 nm, Sigma) diluted (1:100) with PBS + 1% BSA. The grids were then washed 4 × 5 min in PBS + 1% BSA, followed by the same solution without BSA (4 × 5 min). The samples were finally washed in double distilled water and post-stained for 5 min with 5% aqueous uranyl acetate, followed by 1 min with lead citrate. Samples were visualized and photographed with a Zeiss TEM900 transmission electron microscope. Quantitative analysis of the positive labeling was performed as described by Santiago et al. (2000).

Results and discussion

MeJa treatment increases 13-HPOT metabolizing enzyme(s) in leaf tissue

It is known that 13-HPOT can be metabolized by several enzymes that branch from the oxylipin biosynthetic pathways (Schaller et al. 2005). Such distinct routes are initiated by various enzymes, e.g. hydroperoxide lyase (HPL), peroxygenase, divinylether synthase, and AOS that

catalyzes the committed step for JA production. Since we previously found that the MeJa provoked a remarkable accumulation of a chloroplast-targeted 13-LOX in passion fruit leaves (Rangel et al. 2002), the 13-HPOT metabolizing activity in crude leaf extracts from plants exposed to MeJa vapors was analyzed. Spectrophotometric assays show that 13-HPOT can be metabolized much faster in extracts from MeJa treated plants, when compared to the extracts from the controls (Fig. 1a). Western analysis using

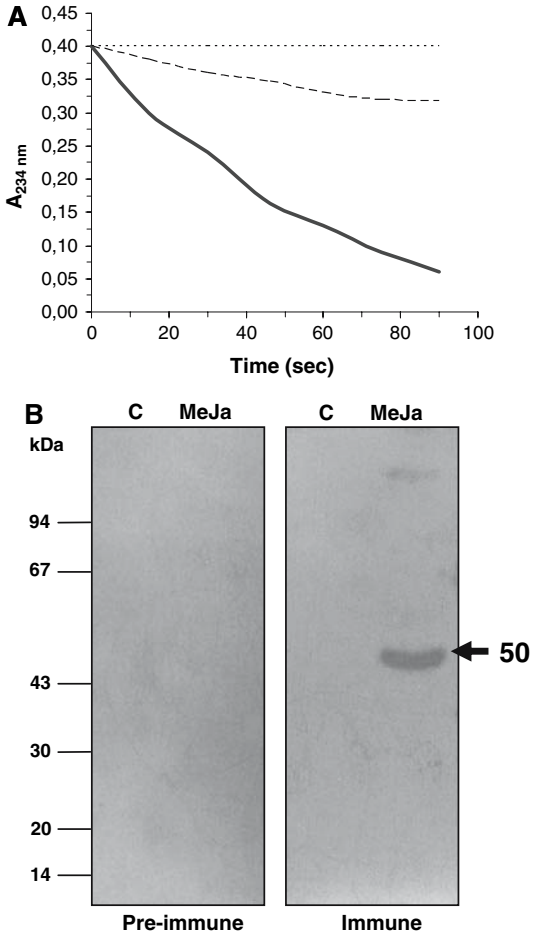


Fig. 1 **a** 13-Hydroperoxy linolenic acid (13-HPOT) metabolizing activity in passion fruit leaves. The presence of 13-HPOT metabolizing enzyme(s) in crude leaf extract from control plants *(dashed line)* and in crude leaf extract from plants exposed to methyl jasmonate (MeJa) during 24 h *(solid line)* was evaluated by spectrophotometric assays. The loss of absorbance of the substrate was monitored at 234 nm. 13-Hydroperoxy linolenic acid was not metabolized in absence of the crude protein extract *(dotted line)* in our assays conditions. The graphic represents a typical result from three independent experiments, **b** western blot analysis: 80 µg of crude leaf protein extract prepared from control plants and MeJa treated plants during 24 h were separated by SDS–PAGE. Proteins were transferred to nitrocellulose membrane and probed with 5,000-fold diluted immune serum raised against tomato AOS (or pre-immune serum). Molecular weight markers are shown on the *left*. In all cases leaves from three plants were pooled for each protein extraction to obtain an average pattern

immune serum against tomato AOS (Howe et al. 2000) revealed that the MeJa treatment caused a strong accumulation of an immune-reactive protein band with ~50 kDa (Fig. 1b), besides a faint band of ~100 kDa. Similar experiments using pre-immune serum showed no immune-reactive bands. The size of the major protein is comparable to that of native plant AOSs in endogenous form or when produced via recombinant systems (Song and Brash 1991; Utsunomiya et al. 2000; Howe et al. 2000; Hughes et al. 2006a; Kongrit et al. 2007). The larger band detected may represent a protein that is not necessarily related to AOS but may be sharing some epitopes. Nevertheless, the western analysis indicated that AOS is contributing to the increased 13-HPOT metabolizing activity. Further biochemical analysis is necessary to ascertain whether MeJa treatment is inducing other 13-HPOT metabolizing enzymes in passion fruit leaves.

Cloning and characterization of P*f*AOS–cDNA

Total RNA used for RT-PCR reactions was isolated from leaves treated with MeJa during 12 h. Based on the conserved regions of known plant AOSs, two degenerated primers were designed to amplify the central region of the AOS coding sequence from passion fruit. Gene-specific primers based on the amplified cDNA fragment were employed to successfully amplify the remaining 5′and 3′ regions ("Materials and methods") of the mRNA. The coding region of 1,512 nucleotides is flanked by a 5′-UTR of 155 nucleotides and a 3′-UTR of 116 nucleotides (GenBank accession number EF601088), and encodes a protein of 504 amino acids with a calculated molecular mass of ~56 kDa. Alignment of the deduced amino acid sequence with that of other AOSs is shown in Fig. 2. P*f*AOS shows 64% identity with *Arabidopsis* AOS (Laudert et al. 1996, Y12636), 65% with guayule AOS (Pan et al. 1995, X78166), 66% with tomato AOS2 (Howe et al. 2000, AF230371), and 67% with flax AOS (Song et al. 1993, U00428). Lower levels of similarity were found with monocot AOSs: 56% identity with rice AOS2 (Agrawal et al. 2002; Haga and Iino 2004, AY062258), and 55% with barley AOS1 (Maucher et al. 2000, AJ250864). Important motifs typical of cytochrome P450, including AOS, such as the I-helix consensus (-G-KIL-), the ETLR motif and the heme binding domain (P-V-NKQCAG) containing the conserved cysteinyl heme ligand for the heme-iron (Cys^{455}), were all identified in P*f*AOS. Thus, according to the established nomenclature for plant AOSs (Howe and Schilmiller 2002; Stumpe and Feussner 2006) based on sequence relatedness, P*f*AOS can be putatively classified as a new member of the CYP74A subfamily of cytochrome P450s. However, a detailed biochemical

characterization of P*f*AOS activity remains to be performed and is essential for a better understanding of the role of this enzyme in fatty-acid signaling in passion fruit. In fact, the importance of such biochemical studies has recently been emphasized and led Hughes et al. (2006b) to propose new rules for the classification of CYP74s enzymes.

Since in our model plant, only 13-LOX activity was detected in crude leaf extracts from wounded or MeJa treated plants (Rangel et al. 2002), it would be reasonable to expect P*f*AOS to metabolize preferentialy 13-hydroperoxides. In addition, AOSs classified as members of the CYP74C subfamily displaying high preference to 9-hydroperoxides have been found in non-photosynthetic tissues, such as tomato roots (Itoh et al. 2002) and potato stolen, sprouts and tubers (Stumpe et al. 2006).

A phylogenetic tree revealed that the P*f*AOS protein is most closely related to dicot AOSs as expected (Fig. 3). Many of these AOSs were characterized as functional chloroplast-targeted proteins involved in JA biosynthesis, reinforcing the role of the putative PfAOS protein in defense signaling. Among monocot proteins P*f*AOS was closer to rice AOS1 which is the only monocot example to date to display a putative chloroplast transit peptide at the *N*-terminal region (Haga and Iino 2004). It is important to note that the location of the early part of the pathway to jasmonate is established as plastidial. Sequence analysis using the ChloroP program (http://www.cbs.dtu.dk/services/ChloroP/) indicates that P*f*AOS contains a typical *N*-terminal transit peptide for targeting the chloroplast, a feature shared with AOSs involved in the biosynthetic route to JA (Schaller et al. 2005).

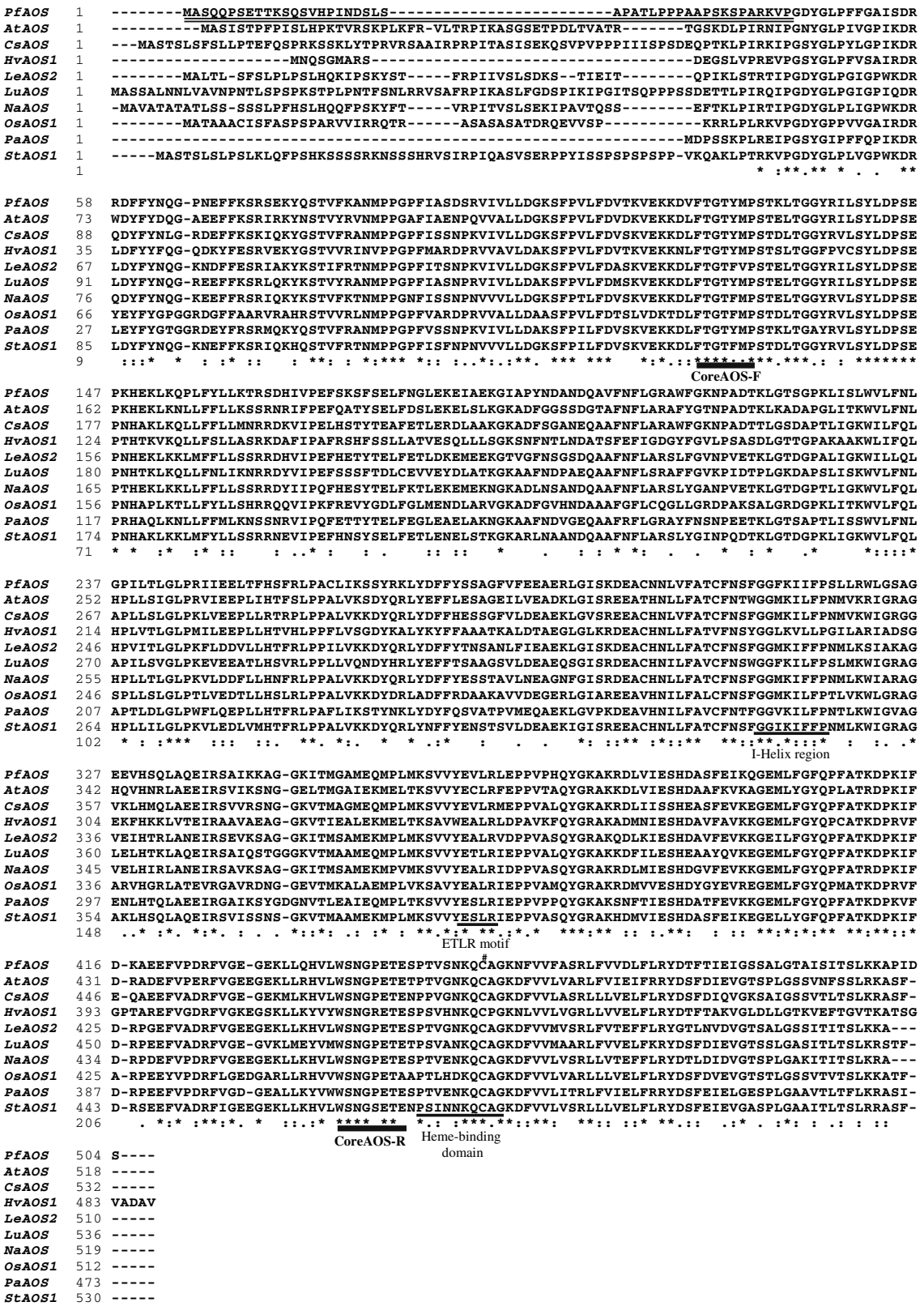
We performed genomic southern analysis to investigate the P*f*AOS gene structure in the passion fruit genome using the P*f*AOS–cDNA central region as a probe. The resulting hybridization pattern, where a single DNA fragment was detected by the probe in each of the three separate DNA digestions with different restriction enzymes is compatible with P*f*AOS, being present as a single copy gene in the passion fruit genome (data not shown). It is in agreement with what has been previously described for AOS genes in plants: one copy in *Arabidopsis*, three copies in tomato (Howe et al. 2000; Sivasankar et al. 2000; Itoh et al. 2002), three copies in barley (Maucher et al. 2000; 2004), and 2–5 copies in rice (Agrawal et al. 2004; Haga and Iino 2004; Chehab et al. 2007).

Mechanical wounding and MeJa treatment induce the accumulation of P*f*AOS transcripts and cystatin in leaf tissue

Passion fruit plants respond to mechanical injury and MeJa treatment, accumulating 13-LOX in leaf tissue (Rangel et al. 2002). To analyze whether P*f*AOS expression could be modulated by the same stimuli northern blot analysis was carried out (Fig. 4). The wound response was observed locally and systemically. Transcripts levels were barely detectable before treatment but increased after 30 min of injury, reaching the highest levels about 9–12 h in the wounded leaves and later decreasing until 24 h, but still considerably higher than the control. The unwounded systemic leaves showed a similar induction pattern, although slightly less intense, which is compatible with the action of a diffusible signal triggering the activation of P*f*AOS expression in distal parts of the plant. In comparison, MeJa elicited a much stronger activation, especially after the first 30 min and lasting the entire time course. This response is consistent with continuous exposure to a powerful signaling molecule. These results are in agreement with different reports showing the wound and/or jasmonate induced accumulation of AOS transcripts in *Arabidopsis*, tomato, barley, tobacco, and rice (Laudert et al. 1996; Howe et al. 2000; Maucher et al. 2000; Ziegler et al. 2001; Agrawal et al. 2002). The northern blot results suggest that the P*f*AOS transcripts are positively regulated by endogenous jasmonate(s) that occurs under wound-stress signal transduction pathway.

Considering that the wound response also includes induction of gene expression of defensive proteins responsive to jasmonates, the accumulation of proteinase inhibitors in leaf tissue under the same conditions described above was also investigated. Figure 5 shows that such treatment caused an increase of papain inhibitors in crude leaf extracts. In parallel, an immuno-reactive polypeptide of about 60 kDa was detected by western assays, using immune serum against tomato cystatin (Madureira et al. 2006). The amount of the recognized protein was consistent with the in vitro inhibitory assays, although the MeJa

Fig. 2 Comparison of deduced amino acid sequences of plant AOSs. ▶ The predicted passion fruit P*f*AOS sequence is compared with the primary structures of AOS from *Arabidopsis thaliana* (AtAOS, Y12636); *Citrus cirensis* (CsAOS, AY243478.1); *Hordeum vulgare* (HvAOS1, AJ250864); *Lycopersicon esculentum* (LeAOS2, AF230371); *Linum usitatissimum*, (LuAOS, U00428-1); *Nicotiana attenuate* (NaAOS, AJ295274); *Oriza sativa* (OsAOS1, AB116527); *Parthenium argentatum* (PaAOS, A56377); *Passiflora flavicarpa* (P*f*AOS, EF601088) and *Solanum tuberosum* (StAOS1, AJ457080). CoreAOS-F and CoreAOS-R indicate the conserved amino acid residues in plant AOSs based on which the degenerate primers were designed and used to amplify the P*f*AOS–cDNA core sequence (see "Materials and methods"). The *double line* denotes a chloroplast transit peptide. The C-terminal heme-binding domain consensus (the heme-binding C is marked by *hash symbol* and the well conserved, I-helix region and ETLR motifs of the CYP74A enzymes are *underlined*. *Asterisks* denote the strictly conserved amino acid residues among monocot and dicot AOSs. Amino acid similarity (**.** or **:**) is based on the CLUSTAL-W convention, and *dashes* indicate gaps introduced for alignment



treatment could have induced additional cystatin isoforms. These results indicate that the wound response in passion fruit leads to the systemic accumulation of cystatin, a defensive protein with potential action against various pests and predators such as insects, nematodes, fungi, and slugs (Martinez et al. 2005).

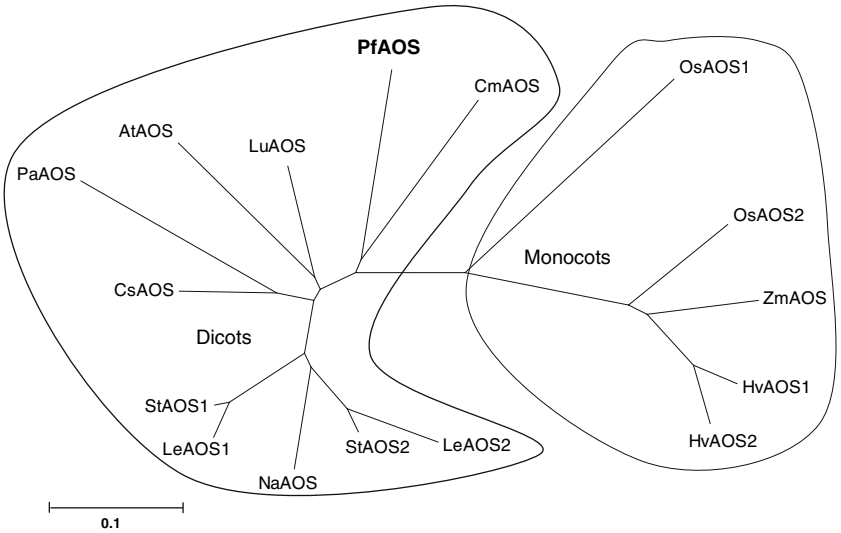


Fig. 3 An unrooted phylogenetic tree of deduced amino acid sequences of plant AOSs. Amino acid sequences were aligned using Clustal W. The phylogenetic and molecular evolutionary analysis were performed using MEGA version 4. P*f*AOS is indicated in bold. The tree was obtained by the neighbor-joining method using 1,000-bootstrap replicates. The scale represents 0.1 substitutions per site. Plant species and accession numbers: *Arabidopsis thaliana* (AtAOS, AY128755-1); *Cucumis melo* (CmAOS, AF081954-1); *Citrus sinensis* (CsAOS, AY243478); *Hordeum vulgare* (HvAOS1, AJ250864; HvAOS2, AJ251304); *Lycopersicon esculentum* (LeAOS1, AJ271093; LeAOS2, AF230371); *Linum usitatissimum* (LuAOS, U00428-1); *Nicotiana attenuate* (NaAOS, AJ295274); *Oriza sativa* (OsAOS1, AB116527; OsAOS2, AY062258); *Parthenium argentatum* (PaAOS, A56377); *Passiflora flavicarpa* (PfAOS, EF601088); *Solanum tuberosum* (StAOS1, AJ457080; StAOS2, AJ457081); and *Zea mays* (ZmAOS, AAR33048-1)

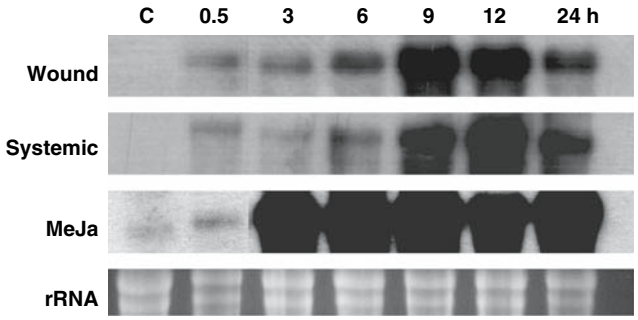


Fig. 4 Time course analysis of P*f*AOS gene expression in response to mechanical injury and MeJa treatment. Lower damaged leaves *wound*, upper undamaged leaves *systemic* and MeJa treated leaves *MeJa* were harvested at specific intervals over a 24 h period. Total RNA was extracted and 15 μg analyzed by northern blotting using a radiolabelled probe (P*f*AOS–cDNA central region). Ethidium bromide-stained rRNA is shown as loading control. For each time point six plants were pooled for RNA extraction to obtain an average response

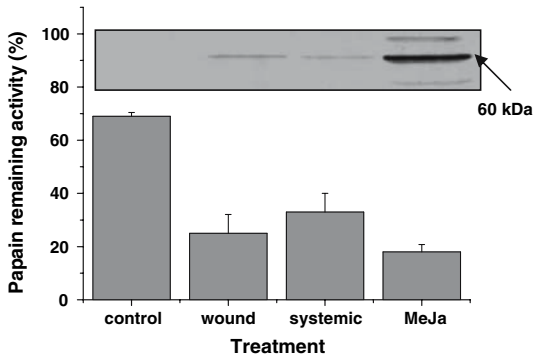


Fig. 5 Papain inhibitory activity in passion fruit leaves. From each sample 70 μg of crude leaf extract were combined with 1 μg of papain and the proteolytic activity evaluated using BANA as substrate. Samples were analyzed 24 h after stimuli. Data are a mean of three independent experiments ratios of standard error to mean were 7% or less. Insert: western blot analysis of crude leaf proteins extracts. *Left to right* control leaves; wounded leaves; systemic leaves; MeJa treated leaves. For each sample 80 μg of total protein were loaded. Proteins were probed with immune serum against tomato cystatin 5,000-fold diluted. In all cases leaves from three plants were pooled for each protein extraction to obtain an average pattern

Subcellular localization of P*f*AOS protein within the chloroplast

Ultrastructural electron microscopy was employed to investigate the subcellular localization of P*f*AOS in leaf tissue via immuno-gold labeling experiments. It should be noted that the possible interference regarding the larger protein identified in the western results may be negligible due its weak signal (Fig. 1b).

Ultrathin sections from control and MeJa-treated leaves probed with pre-immune serum showed no labeling (data not shown). Ultrathin sections from MeJa-treated leaves probed with immune serum against tomato AOS showed positive labeling in chloroplasts of mesophyll cells

(Fig. 6a) with minor labeling in the cytosol. Within the chloroplast, gold particles were found associated with stroma and thylakoid membranes. In contrast, ultrathin sections from control leaves probed with immune serum failed to show positive labeling (Fig. 6a). Quantitative analysis revealed a stronger labeling in the stroma and more variation in the labeling found in the thylakoid membranes (Fig. 7). However, it is unclear whether the labeling pattern reflects the presence of one or more AOS isoforms, which could be distributed unevenly between these structures. Our previous immuno-gold labeling studies addressing the subcellular localization of a MeJa-inducible 13-LOX revealed a different distribution since this enzyme was found almost exclusively in the stroma (Rangel et al. 2002). Therefore, the immunocytochemistry results obtained for P*f*AOS not only corroborate its predicted localization, but can also place this enzyme next to a putative substrate source provided by a 13-LOX.

AOS from other plants were also described to accumulate in the chloroplast. By means of in vitro chloroplast import assays, tomato AOS was found peripherally associated almost exclusively with the inner membrane of the organelle, with the bulk of the protein oriented towards the stroma (Froehlich et al. 2001). Using different approaches including ultrastructural analysis and immuno-gold labeling Farmaki et al. (2007) demonstrated the chloroplastic location of potato AOS in unwounded leaf tissue. The potato AOS was found preferentially and strongly bound to the thylakoid membranes. Such results indicate the existence of variations on the distribution of AOSs within the chloroplast of different plant species as well as differences in the nature of association with the organelle's structures.

Recently, Hughes et al. (2006a) demonstrated by in vitro analysis that the purified recombinant AOS from *Arabidopsis*, has its enzymatic parameters modulated by detergent micelle association. Authors showed that, this previously described 13-AOS can indeed display dual substrate specificity (e.g. 9- and 13-AOS activity), but with low activity in the absence of detergent micelles. In contrast, the micelle-bound form displays highly increased 13-AOS activity, but still considered retaining 9-AOS activity.

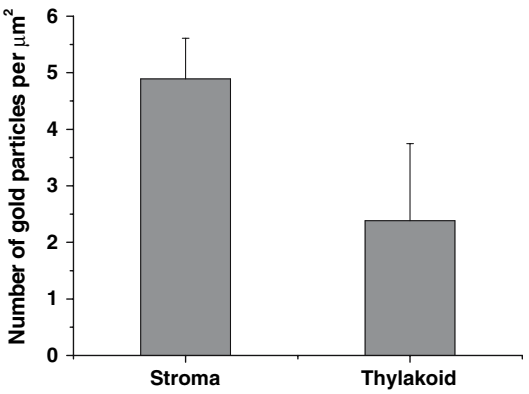


Fig. 7 Quantitative analysis of colloidal gold particles within chloroplasts. Particles were counted from over 20 random chloroplasts from three plants

However, similar studies with purified recombinant HPL from *Medico truncatula*, another cytochrome P450 enzyme involved in the oxylipin metabolism showed that the micelle association affected enzyme activity, but not substrate specificity (Hughes et al. 2006b). The spatial localization observed for P*f*AOS within the chloroplast, e.g. in the stroma and thylakoid membranes may indicate an in vivo activity in both the water-soluble as well as the membrane-bound state. Such a picture raises interesting questions in relation to the mechanisms underlying the mobilization from a membrane-associated to a free soluble form. In addition, it is possible that the variation of the membrane-bound state of AOS could affect the enzymatic parameters in vivo, and such plasticity could help plants adjust their oxylipin profile in order to better respond to environmental challenges.

Conclusions

In the present study, we characterized the cDNA from a novel AOS-encoding gene that might be involved in passion fruit defense signaling against insect pests. Additional biochemical and genetics studies are required to fully understand the AOS function in such defense mechanisms.

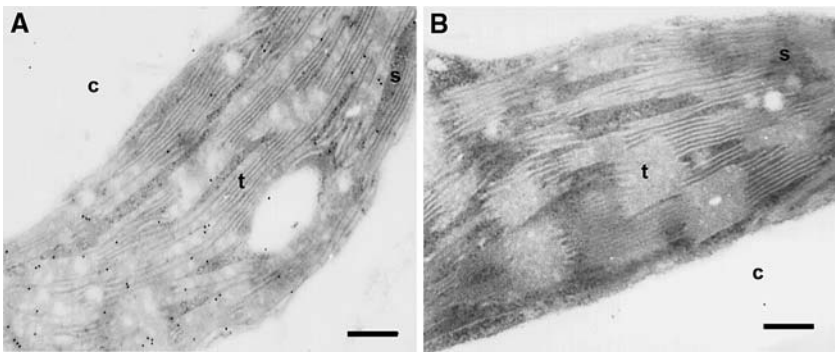


Fig. 6 Immunocytochemical localization of the MeJa-induced AOS protein in passion fruit leaves. **a** Ultrathin-section of MeJa exposed leaf collected 24 h after treatment, *bar* represent 250 nm and **b** ultrathin-section of control leaf, *bar* represent 150 nm. Stroma *s*, thylakoid *t* and cytosol *c* are indicated

The results presented in this work contribute to the information available concerning the importance of cytochrome P450 enzymes for the oxylipin metabolism and signaling transduction in plant defense.

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