Enzymes of Jasmonate Biosynthesis Occur in Tomato Sieve Elements

Bettina Hause 1, Gerd Hause 2, Claudia Kutter 3, Otto Miersch 3 and Claus Wasternack 3, 4

1 Department of Secondary Product Metabolism, Institute for Plant Biochemistry, Weinberg 3, D-06120 Halle, Germany
2 Biocenter, University of Halle-Wittenberg, Weinbergweg 22, D-06120 Halle, Germany
3 Department of Natural Product Biotechnology, Institute of Plant Biochemistry, Weinberg 3, D-06120 Halle, Germany

The allene oxide cyclase (AOC) is a plastid-located enzyme in the biosynthesis of the signaling compound jasmonic acid (JA). In tomato, AOC occurs specifically in ovules and vascular bundles [Hause et al. (2000) Plant J. 24; 113]. Immunocytochemical analysis of longitudinal sections of petals and flower stalks revealed the occurrence of AOC in companion cells (CC) and sieve elements (SE). Electron microscopic analysis led to the conclusion that the AOC-containing structures of SE are plastids. AOC was not detected in SE of 35S::Actin sense plants. The enzymes preceding AOC in JA biosynthesis, the allene oxide synthase (AOS) and the lipoxygenase, were also detected in SE. In situ hybridization showed that the SE are free of AOC-mRNA suggesting AOC protein traffic from CC to SE via plasmodesmata. A control by in situ hybridization of AOS mRNA coding for a protein with a size above the exclusion limit of plasmodesmata indicated mRNA in CC and SE. The data suggest that SE carry the capacity to form 12-oxo-phytodienoic acid, the unique precursor of JA. Together with preferential generation of JA in vascular bundles [Stenzel et al. (2003) Plant J. 33: 577], the data support a role of JA in systemic wound signaling.

Keywords: Enzymes in JA biosynthesis — Immunocytochemistry — Sieve elements — Tomato.

Abbreviations: α-LeA, α-linolenic acid; AOC, allene oxide cyclase; AOS, allene oxide synthase; CC, companion cells; 13-HPOT, 13-hydroperoxy-octadecatrienoic acid; JA, jasmonic acid; JAME, JA methyl ester; 13-LOX, 13-lipoxygenase, OPDA, 12-oxo-phytodienoic acid; SE, sieve elements.

Jasmonic acid (JA) and its methyl ester (JAME) collectively called jasmonates, are members of a lipid-based signaling cascade originating from polyunsaturated fatty acids such as α-linolenic acid (α-LeA). Together with their precursor 12-oxo-phytodienoic acid (OPDA), these compounds are signals in plant responses to biotic and abiotic stresses and in plant development (Wasternack and Hause 2002). A common property of numerous plant stress responses is the elevation of OPDA and jasmonate levels followed by expression of jasmonate-responsive genes. Among them are genes that code for enzymes of JA biosynthesis thus implicating a positive feed-back loop.

The biosynthesis of JA is initiated by insertion of molecular oxygen into position 13 of α-linolenic acid (α-LeA) catalyzed by a 13-lipoxygenase (13-LOX) (Feussner and Wasternack 2002). The LOX product (13S)-hydroperoxy (9Z,11E,15Z) octadecatrienoic acid (13-HPOT) is converted by an allene oxide synthase (AOS) to highly unstable allene oxide which in turn can rapidly decay in aqueous solution by chemical hydrolysis to α- and γ-ketols and racemic OPDA. In the presence of the allene oxide cyclase (AOC), exclusive formation of the cis-(+)-enantiomer (9S,13S) of OPDA occurs. This enantiomer carries the structure of the naturally occurring (+)-7-iso-JA, which is generated from an OPDA reduction and three β-oxidative steps.

The initial substrate of JA biosynthesis is α-LeA and originates from chloroplast membranes. A recently characterized JA-deficient mutant of Arabidopsis, was found to be affected in a chloroplast-located phospholipase A1 (Ishiguro et al. 2001). Subsequent enzymes of the first half of JA biosynthesis including the 13-LOX, the AOS and the AOC, carry putative target sequences for plastid import, and their location within chloroplasts was shown by immunocytochemical analysis (Feussner et al. 1995, Maucher et al. 2000, Ziegler et al. 2000) or import studies (Bell et al. 1995, Froehlich et al. 2001). OPDA reduction was localized in peroxisomes (Stintzi and Browse 2000, Strassner et al. 2002), where the final β-oxidation steps are assumed to occur.

The occurrence of enzymes of JA biosynthesis and the ability to form JA and related compounds were observed in various tissues including flower organs (Hause et al. 2000), leaves (Wasternack and Hause 2002), roots (Hause et al. 2002), as well as mesocotyl (Maucher et al. 2000). The latter examples indicate that JA biosynthesis is not dependent on photosynthetically active chloroplasts. There is, however, a remarkable tissue-specificity in the occurrence of enzymes of JA biosynthesis such as AOS and AOC. In barley, AOS is expressed in young convoluted leaves, in the first internode and in the scutellar node, all exhibiting elevated JA levels.

4 Corresponding author: E-mail, cwastern@ipb-halle.de; Fax, +49-345-5582-1219.
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(Maucher et al. 2000). In tomato, the AOC, a protein of 26 kDa, was detected by immunocytochemical analysis exclusively in vascular bundles of petioles, petiolules and main veins of leaves, in the bundle sheet of the minor veins and in ovules of flower buds (Hause et al. 2000).

The phloem, composed of enucleate sieve elements (SE) which are intimately connected with companion cells (CC), functions in long distance transport of signaling molecules and pathogens and for nutrient reallocation (Oparka and Turgeon 1999). More than 200 proteins were found in fully developed SE ranging from 2 to 200 kDa. Among them are the abundant P-proteins (Golecki et al. 1999), proteinases (Mehta et al. 1996), proteinase inhibitors (Xu et al. 2001) and protein kinases (Yoo et al. 2002). Only some SE-located proteins were found in plastids. Sucrose transporters could be co-localized in plasma membranes of SE and may function by oligomerization (Reinders et al. 2002). Recent presence and activity of a complete antioxidant defense system was detected in mature SE (Walz et al. 2002). This example emphasizes that in SE circuit functions of nucleate cells are kept.

Direct analysis of phloem sap composition could be performed with only few plants due to sealing of the wounded phloem. In Cucurbita, Ricinus, Perilla or Oryza, phloem sap could be sampled directly or by use of aphids followed by complex analysis including matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Haebel and Kehr 2001, Hoffmann-Benning et al. 2002, Nakamura et al. 1995). In most cases location of proteins in the SE was identified via immunocytological techniques. Here, we show by means of immunocytochemical analysis that enzymes of JA biosynthesis such as LOX, AOS and AOC are located within SE. Using in situ hybridizations, AOC-mRNA was found exclusively in CC, whereas AOS mRNA was found in both cell types suggesting AOC transport via plasmodesmata into SE. The data implicate a role in systemic wound signaling.

Lycopeisson esculentum Mill. cv. Lukullus plants were grown as described (Wasternack et al. 1998). Identical cultivation conditions were used for the homozygous transgenic line of the T2 generation carrying the tomato cDNA for AOC in antisense orientation under the control of the 35S promoter. Field grown Plantago major plants were used for mechanical isolation of intact phloem from petioles. For extraction and quantitative analysis of JA and OPDA, tissues (0.5 g FW) were frozen in liquid nitrogen, homogenized in a mortar and 80% (v/v) methanol. For quantification Purification, fractionation and quantitative analysis of OPDA and JA by HPLC and GC-MS were performed as described (Hause et al. 2000, Kramell et al. 2000).

Small pieces of petioles or flower stalks of 6-week-old tomato plants were harvested and fixed with 3% (w/v) paraformaldehyde in PBS (135 mM NaCl, 3 mM KCl, 1.5 mM KH₂PO₄, 8 mM Na₂HPO₄). After dehydration by a graded series of ethanol, material was embedded in paraplast (Sigma, Deisenhofen, Germany) or polyethylene glycol (PEG, Merck, Darmstadt, Germany). For in situ hybridization cross-sections of paraplast-embedded material (10 μm thick) were mounted on poly-L-lysine-coated slides, deparaffinized and rehydrated. In situ hybridization was performed as described (Maucher et al. 2000) using DIG-labeled antisense and sense RNA probes obtained from tomato AOC cDNA or tomato AOS2 cDNA (Howe et al. 2000) by in vitro transcription.

Immunocytochemical analysis was performed as described (Hause et al. 2000) using longitudinal sections (2 μm thick) from PEG-embedded tissues. Samples were analyzed with an anti-LOX-antibody raised against the lipid body LOX of cucumber (Feussner et al. 1995) (dilution of 1:500), an anti-AOS-antibody raised against recombinant tomato AOS (Howe et al. 2000) (dilution 1:1,000) and an anti-AOC-antibody raised against recombinant tomato AOC (Ziegler et al. 2000) (dilution 1:2,000), followed by treatment with anti-rabbit IgG-conjugated with Alexa 488 (Molecular Probes Eugene, OR, U.S.A.) as secondary antibody. To visualize nuclei, sections were subsequently stained with the DNA dye 4,6-diamidino-2-phenylindole (DAPI, SIGMA Chemical Co., St. Louis, MO, U.S.A.). Stained sections were analyzed with an epifluorescence microscope “Axioskop” (Carl Zeiss, Jena, Germany) using the bright field mode (in situ hybridization) and fluorescence mode with the proper filter combinations (immunocytochemical analysis), respectively.

For electron microscopic analysis petioles were fixed in 3% (v/v) sodium cacodylate-buffered glutaraldehyde (pH 7.2), post-fixed with 1% (w/v) OsO₄ solution, dehydrated in an ethan-alcohol series and embedded in epoxy resin (Spurr 1969). Ultrathin-sections (90 nm) were stained with uranyl acetate/lead citrate and observed with an EM 900 transmission electron microscope (Zeiss, Oberkochen, Germany).

The phloem-specific occurrence of AOC protein in tomato (Hause et al. 2000) and the preferential occurrence of OPDA and JA in main veins compared to leaf lamina (Stenzel et al. 2003) prompted us to inspect localization of AOC protein by immunolabeling in more detail including putative occurrence in SE. Since it is impossible to cut mechanically main vein-containing leaf areas free of the surrounding mesophyll cells, we inspected for comparison P. major leaf petioles from which phloem can be easily isolated. In cross-sections the isolated phloem was free of mesophyll tissue (data not shown). This corresponds to the fact that a phloem-specific cDNA library could be generated from isolated phloem of P. major (Stadler et al. 1995). The isolated phloem of P. major contained 13.6 nmol OPDA (g FW)⁻¹ and 5.8 nmol JA (g FW)⁻¹, whereas the whole leaf tissue contained 0.1 nmol OPDA (g FW)⁻¹ and 0.06 nmol JA (g FW)⁻¹. The abundant occurrence of the AOC product OPDA in the phloem of P. major prompted us to inspect localization of AOC in vascular bundles of tomato in more detail by immunolabeling with an antibody raised against recombinant...
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AOC of tomato. The anti-AOC antibody did not show cross-reactivity with other proteins in the immunoblot analysis of total leaf extracts (Hause et al. 2000). Together with the fact that the AOC is encoded by a single copy gene in tomato (Ziegler et al. 2000), specific detection of AOC protein in the immunocytochemical analysis is highly probable. Immunocytochemical analysis of longitudinal sections of flower stalks and petioles revealed the occurrence of AOC in CC and SE (Fig. 1). SE were identified by the presence of sieve plates, lack of nuclei and the tube-like shape. All SE contained small but strong fluorescent dots indicating occurrence of the AOC protein. In contrast to SE of wild-type flower stalks, the AOC protein was not detectable in SE of 35S::AOCantisense plants (Fig. 3), thereby supporting that AOC protein was detected with the anti-AOC antibody. The neighboring CC identified by DIC-imaging and DAPI-staining of nuclei exhibited strong label in plastids (Fig. 1).

The dots within the SE appeared exclusively parietal. Since the tomato AOC carries a chloroplastic transit peptide and the AOC protein was localized immunocytochemically in chloroplasts of tomato mesophyll cells (Ziegler et al. 2000), we assumed that the dots belong to SE plastids. The size of the labeled structures is about 1 μm (Fig. 1A). Electron microscopic analysis revealed that in SE only plastids have a diameter of about 1 μm (Fig. 2). Other organelles such as mitochondria or remnants of the cytoplasm are smaller (Fig. 2B). Plastids of SE identified for many different species were subdivided into S-type plastids, containing mainly starch inclusions, and P-type plastids, containing main protein inclusions (Behnke 1981). Due to the absence of starch-granules within the SE, we assume a P-type in tomato. A more detailed localization of AOC in the SE plastids by means of immunogold technique failed due to the low amount of AOC protein in SE.

Immunocytochemical inspection of LOX and AOS protein revealed that both proteins are also located in SE (Fig. 3). Although there was some cross-reactivity of the anti-AOS antibody and the anti-LOX-antibody presumably with callose of sieve plates, fluorescent labeling in SE was obvious. With the anti-AOS antibody one band was found in the immunoblot analysis of total leaf extracts of untreated tomato leaves (Howe et al. 2000). Thus, it is probable that this antibody recognizes both highly homologous 13-AOS forms known to occur in tomato leaves (Howe et al. 2000, Sivasankar et al. 2000). Using the anti-LOX antibody, one band was detected in the immunoblot analysis of total leaf extracts of untreated tomato leaves (Wasternack et al. 1998). It cannot be excluded that more than one LOX form is detected in the immunocytochemical analysis shown here, since at least two 13-LOX forms of the five LOX forms encoded in tomato occur in tomato leaf plastids (Feussner and Wasternack 2002). The label indicative for LOX protein and AOS protein is also parietally located, but not exclusively as found with the anti-AOC antibody (Fig. 3).

In phloem sap, specific populations of mRNAs have been clearly detected (Oparka and Turgeon 1999). Among them were virus RNA and mRNA coding for the sucrose transporter SUT1. There are hints suggesting that SE-located mRNAs are translated (Kühn et al. 1997). However, most of mRNAs detected in SE were thought to be linked to long distance traffic
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Haywood et al. (2002). Here, we inspected the location of AOC-mRNA by in situ hybridization of cross-sections of flower stalks (Fig. 4). As shown by an overall view of a cross-section, AOC-mRNA was clearly detectable in the bicolateral vascular bundles, preferentially in the inner part, and in the epidermal layer. Among SE and CC inspected at higher magnifications, the AOC-mRNA occurred exclusively in CC indicated by number and position around the triangle-shaped SE which are also marked by the thick cell wall (Fig. 4D). As a control we detected, by in situ hybridization, AOS mRNA which codes for a protein larger than the exclusion limit of plasmodesmata. Among inspected SE and CC most SE revealed clearly occurrence of AOS mRNA (Fig. 4E).

The occurrence of AOC protein in CC and SE plastids accompanied with the exclusive location of AOC mRNA but not of AOS mRNA in CC suggest a traffic of AOC protein into SE via plasmodesmata. To date there is little doubt that proteins synthesized in CC are transferred into SE (Oparka and Turgeon 1999). Although the exact mechanism is unclear, green 27 kDa fluorescent protein (GFP) synthesized in CC was shown to enter the SE and to migrate within the phloem (Imlau et al. 1999). The size exclusion limit of plasmodesmata was shown to be at about 50 kDa in sink leaves and decreased by a developmental switch from non-branched to branched plasmodesmata (Oparka et al. 1999). The 26 kDa AOC protein is clearly within the range suggesting its traffic via plasmodesmata.

The occurrence of AOC in SE and CC, together with the preceding enzymes in JA biosynthesis, LOX and AOS, and the preferential generation of JA and OPDA in main veins of tomato leaves (Stenzel et al. 2003) might be of remarkable consequence for the wound response of tomato plants. Here, JA and the 18 amino acid peptide systemin which is also encoded in vascular bundles (Jacinto et al. 1997). Based on data on the local wound response (Ryan 2000, Ryan et al. 2002) and on transgenic tomato plants expressing AOC in sense and antisense orientations, an amplification in wound signaling by a systemin-dependent AOC activation and a JA-inducible prosystemin.
gene expression both taking place in vascular bundles was proposed (Stenzel et al. 2003). The outcome of this amplification would be a spatial and temporal coincidence of the generation of JA. Recent grafting experiments (Li et al. 2002) strengthen the hypothesis that a rapid and sufficient elevation of JA in the local leaf may trigger the systemic wound response (Ryan and Moura 2002). JA or OPDA levels (Herde et al. 1996, Landgraf et al. 2002) may elevate systemically following local wounding and pathogen attack, respectively, or might be constant (Strassner et al. 2002), but JA perception in the systemic leaf is essential (Li et al. 2002). Loading of JA into SE or its formation within the SE may support a systemic response which finally leads to expression of plant defense genes such as those coding for proteinase inhibitors (Ryan 2000). Analysis of tomato phloem sap will help to answer open questions in JA-mediated wound signaling.

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft (DFG) (project C5, SFB 363). We thank Prof. I. Feussner (University of Goettingen, Germany) for supply of the LOX antibody, Prof. G. Howe (University of Michigan, U.S.A.) for supply of the tomato AOS antibody, Prof. L. Varin (Montreal, Canada) for critical reading and Mrs. C. Dietel for typing of the manuscript.

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(Received December 2, 2002; Accepted March 25, 2003)