Expression of Allene Oxide Cyclase and Accumulation of Jasmonates during Organogenic Nodule Formation from Hop (Humulus lupulus var. Nugget) Internodes

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A crucial step in the biosynthesis of jasmonic acid (JA) is the formation of its stereoisomeric precursor, cis-(+)-12-oxophytodienoic acid (OPDA), which is catalyzed by allene oxide cyclase (AOC, EC 5.3.99.6). A cDNA of AOC was isolated from Humulus lupulus var. Nugget. The ORF of 765 bp encodes a 255 amino acid protein, which carries a putative chloroplast target sequence. The recombinant protein without its putative chloroplast target sequence showed significant AOC activity. Previously we demonstrated that wounding induces organogenic nodule formation in hop. Here we show that the AOC transcript level increases in response to wounding of internodes, peaking between 2 and 4 h after wounding. In addition, Western blot analysis showed elevated levels of AOC peaking 24 h after internode inoculation. The AOC increase was accompanied by increased JA levels 24 h after wounding, whereas OPDA had already reached its highest level after 12 h. AOC is mostly present in the vascular bundles of inoculated internodes. During prenodule and nodule formation, AOC levels were still high. JA and OPDA levels decreased down to 10 and 118 pmol (g FW)−1, respectively, during nodule formation, but increased during plantlet regeneration. Double immunolocalization analysis of AOC and Rubisco in connection with lugol staining showed that AOC is present in amylloplasts of prenodular cells and in the chloroplasts of vacuolated nodular cells, whereas meristematic cells accumulated little AOC. These data suggest a role of AOC and jasmonates in organogenic nodule formation and plantlet regeneration from these nodules.

Keywords: Allene oxide cyclase — Humulus lupulus — Jasmonates — Organogenic nodule.

Introduction

Oxylipins such as jasmonates and octadecanoids are oxygenated compounds derived from polyunsaturated fatty acids. They are considered to be signals in growth and development, in responses to biotic and abiotic stresses, in the formation of volatiles functioning as aromas or in plant–plant and plant–insect communication (Wasternack and Hause 2002).

Jasmonates include jasmonic acid (JA) and its methyl ester (JAME), whereas octadecanoids comprise 12-oxophytodienoic acid (OPDA) and its derivatives. These compounds are synthesized by one of the seven different branches of the lipoxgenase (LOX) pathway, which is initiated by the LOX-catalyzed oxygenation of α-linolenic acid (α-LeA) (Feussner and Wasternack 2002). The insertion of oxygen can occur at position 13 by a 13-LOX or at position 9 by a 9-LOX. The 13-LOX product with α-LeA as a substrate is a hydroperoxide [13S-hydroperoxy-(9Z, 11E, 15Z)-octadecatrienic acid, 13-HPOT]. This compound, 13-HPOT can be converted by allene oxide synthase (AOS) into an unstable allene oxide (12,13-epoxylinolenic acid), which is cyclized by an allene oxide cyclase (AOC) to cis-(+)-OPDA (9S, 13S) carrying the enantiomeric structure of the naturally occurring (+)-7-iso-JA. The reduction of the cyclopentenone ring is catalyzed by an OPDA reductase (OPR). This is followed by three rounds of β-oxidation leading to (+)-7-iso-JA, which equilibrates to the more stable (−)-JA.

All the enzymes leading to JA biosynthesis have been cloned, and in the case of β-oxidation the fatty acid β-oxidation enzymes have been shown to function in the final steps of JA biosynthesis (Schilmiller and Howe 2005). Several cDNAs coding for different 13-LOXs and AOS isoenzymes have been characterized in several plant species (Feussner and Wasternack 2002). The first full-length cDNA coding for AOC was isolated from tomato. It carries a putative chloroplast transit peptide sequence (Ziegler et al. 2000). Further cDNAs coding for AOC have been characterized from various plant species such as Arabidopsis thaliana (Stenzel et al. 2003b), barley (Maucher et al. 2004) and Vitis vinifera (H. Cardoso personal communication).
Among the OPRs known so far, only OPR3 has been shown to reduce efficiently the natural cis-(-)OPDA as analyzed for Arabidopsis and tomato (Schaller et al. 2000, Strassner et al. 2002).

Most plants analyzed so far accumulate JA transiently within the first hour upon wounding but also in response to other external stimuli (Stenzel et al. 2003a). GC-MS analysis revealed that the rise in JA is preceded by a more pronounced rise in octadecanoids such as OPDA, as shown for elicited cell suspension cultures (Parchment et al. 1997), osmotic stress (Kramell et al. 2000), wounding or touch of tendrils (Stelmach et al. 1998). The rise in the levels of jasmonates and OPDA is followed by the induction of several genes such as those coding for proteinase inhibitors, thionins, defensins and enzymes involved in the biosynthesis of phytoalexins, alkaloids and monoterpenes (review, Wasternack and Hause 2002). Enzymes involved in the biosynthesis of JA are also induced by exogenous application of JA (Laudert and Weiler 1998, Maucher et al. 2000). JA action also leads to down-regulation of house-keeping proteins such as Rubisco, which may be part of its senescence-promoting effect (Creelman and Mullet 1997).

Beside jasmonates, OPDA has also been shown to possess signaling properties, e.g. in volatile formation (Koch et al. 1999), in tendril coiling (Stelmach et al. 1998), and in plant pathogen interactions (Stintzi et al. 2001). Recently, cycloartenone compounds such as OPDA were shown to activate or to repress gene transcription through the activities of a conserved electrophilic part of OPDA (Alméras et al. 2003). The profiles of oxylipins were found to be different in various plant species, which led to the suggestion that such an ‘oxylipin signature’ may mediate plant-specific JA/OPDA-related responses (Weber et al. 1997).

The availability of cDNAs coding for JA biosynthetic enzymes allowed the study of the physiological effects of JA by transgenic approaches. In developmental processes or stresses (e.g. wounding) a tight correlation between the expression of genes coding for JA biosynthetic enzymes and elevation of JA and OPDA was found (Hause et al. 2000, Maucher et al. 2000); this was at least partially reflected in AOS promoter activity studies (Kubigsteltig et al. 1999). In most cases, however, constitutive overexpression of AOS (Laudert et al. 2000) or AOC (Stenzel et al. 2003a) did not lead to constitutive elevation of JA levels in leaves. In contrast, elevated JA levels were found in such overexpression lines in comparison with wild-type plants upon wounding of leaves. This indicates regulation of the JA biosynthesis by substrate availability. During tomato development AOC is expressed in the vascular bundles of roots, stems, flower stalks and leaves and in the ovules of young flowers (Hause et al. 2000). These tissue-specific transcript levels were tightly correlated with high levels of JA, JAME, JA amino acid conjugates and those of octadecanoids. Furthermore, individual flower tissues contained unique ratios of the various jasmonates and octadecanoids. This suggested specific roles for AOC and octadecanoid/jasmonate profiles during flower development and stress responses. In A. thaliana increased levels of AOC during seedling development were detected and flowers contained AOC protein in ovules and vascular bundles shortly before opening (Hause et al. 2003b).

Although morphogenetic processes such as somatic embryogenesis and organogenic nodule (ON) formation play a pivotal role in plant biotechnology, little is known about the processes involved in their induction and development. Nodular structures have been studied in several plant species and were found to be an additional morphogenetic pathway useful for regeneration strategies, automated micropropagation and genetic transformation for desirable characteristics (McCown et al. 1988). Organogenic nodule formation and the involvement of wounding in their induction have been described previously in hop (Humulus lupulus L.) (Fortes and Pais 2000, Fortes et al. 2004, Silva et al. 2004).

Hop is an economically important plant known for the production of acids and essential oils used in brewing. In this study, we show data for this species suggesting a link of wound-induced organogenic nodule formation and plantlet regeneration with AOC expression and OPDA/JA formation.

### Results

**Cloning of AOC and AOS homologs from hop and expression of hop AOC in Escherichia coli**

The protein-coding region of the putative hop AOC cDNA encompasses 765 bp, corresponding to a protein of 255 amino acids and with an isoelectric point of 8.58. The calculated molecular mass was 27.98 kDa. Sequence alignment of the putative protein indicates high degrees of identity (99 and 91%, respectively) to two other hop AOC sequences from H. lupulus cv. Strisselspalt (Fig. 1). There is also high similarity to man- grin, an AOC homologous protein from Bruguiera sexangula (Yamada et al. 2002) and to AOC from Nicotiana tabacum, Medicago truncatula and Lycopersicon esculentum (Fig. 1). This high degree of identity of the sequence presented here with other hop AOCs as well as sequence comparisons with more than 35 AOC-encoding sequences so far available in the database (Stenzel et al. 2003b) encouraged us to designate the hop sequence an AOC. The hop AOC is rich in Ser residues at the N-terminus (30% for the first 50 amino acids), a feature common in chloroplast signal peptides. In silico analysis of the first 100 amino acids was performed with the TargetP V1.0 program (http://www.cbs.dtu.dk/services/TargetP) (Emanuelsson et al. 2000), and a putative chloroplast localization was predicted (cTP 0.973). The putative chloroplast signal peptide corresponds to the first 69 amino acids.

In order to confirm that the putative hop AOC gene indeed codes for an allene oxide cyclase, the complete sequence as well as a truncated version lacking the chloroplast signal peptide, were expressed in E. coli (Fig. 2). After induction, the mature AOC showed significant formation of OPDA (~6.2% OPDA formation per µg protein) whereas the complete...
**Fig. 1** Amino acid sequence alignment of AOC from *H. lupulus* var. Nugget (Acc. No. AY644677; HlAOC), *H. lupulus* var. Strisselspalt (Acc. No. AY687338; HlAOC2 and Acc. No. AY687339; HlAOC3), *N. tabacum* (Acc. No. CAC88341), *L. esculentum* (Ziegler et al. 2000), *M. truncatula* (Acc. No. CAC88340) and mangrin from *Bruguiera sexangula* (Acc. No. BAB21610).
sequence showed lower though significant enzyme activity (∼1.8% OPDA conversion per µg protein).

The partial cDNA sequence coding for an AOS in hop encompasses 900 bp (GenBank Acc. AY745883) and shows a high level of similarity to AOS from *Hevea brasiliensis* and *Solanum tuberosum* at the protein level (72% and 71%, respectively).  

**Wounding of internodes leads to transient AOS mRNA and AOC mRNA accumulation**

Wounding of hop internodes, leading to nodule formation, was characterized within the first hours by transient accumulation of AOS mRNA (Fig. 3A) and AOC mRNA (Fig. 3B). Both mRNAs peaked between 2 and 4 h. During morphogenesis only weak but significant AOC mRNA accumulation could be detected for the first 7 of up to 45 d (Fig. 3C).

**Wounding of internodes leads to AOC protein accumulation**

To test whether the observed AOC mRNA accumulation is accompanied by AOC protein accumulation, identical time points upon wounding were inspected by Western blot analysis. After 8 h of induction, a band of ∼24 kDa corresponding to AOC protein was detected and found to increase for up to 24 h upon wounding (Fig. 4A).

Seven days after inoculation of internodes, and also during prenodule formation, AOC protein was still clearly detectable (Fig. 4B). In nodules, 28 d after inoculation of the internodes, the AOC protein level was lower than that in prenodules. In contrast AOC protein was not significantly detectable during plantlet regeneration from nodules occurring about 45 d after inoculation of internodes (Fig. 4B). The bands shown in Fig. 4A, B are indicative for AOC since the pre-immune serum of the antibody raised against the recombinant tomato AOC did not detect any plant protein.

**Tissue-specific expression of AOC during nodule formation**

In order to inspect the tissue-specific occurrence of AOC protein upon wound-induced nodule formation, cross-sections of internodal explants were analyzed by immunofluorescence microscopy. In non-induced tissues a weak green fluorescent label indicative for AOC was detectable in subepidermal cells and in vascular bundles (Fig. 5A). A similar tissue sample
Fig. 5  Tissue-specific expression of AOC at different stages of ON induction and formation. (A) Confocal imaging of an explant cross-section at day 0 showing anti-AOC labeling at the level of cambial (CB) and cortical (CT) cells. (B) Internode section after 12 h of culture. Anti-AOC labeling is detected in vascular bundles and in some chloroplasts of subepidermic cells. Images are shown with the color code displayed next to the figure, which does not correspond to the color emitted by the fluorochrome. Red spots correspond to the highest intensity of labeling (arrows); blue corresponds to background signal. (C) Confocal imaging of an internode section after 24 h of culture and incubated with pre-immune serum (control). Autofluorescence from vascular cells (VC) and chloroplasts is observed. (D) Bright-field semi-thin section of explant after 7 d on induction medium showing divisions occurring in cambial and cortical cells. (E) Confocal imaging of section showing prenodules (PN) formed from the internode after 15 d in culture and incubated with anti-AOC. Immunofluorescence signal is detected in amyloplasts. (F) Confocal imaging of nodule (N) section incubated with pre-immune serum. No specific labeling was observed. (G) Confocal imaging of a nodule section incubated with anti-AOC. The labeling was detected in amyloplasts. (H) Confocal imaging of a nodule section showing a more intense immunofluorescence signal in regions close to the vascular bundles (VB). (I) Bright-field semi-thin section of a nodule at day 28 showing vascular bundles distributed across the nodule. (J) Bright-field semi-thin section of a nodule at day 45, showing different cell types: vacuolated in inner regions of nodules and meristematic at periphery. (K) Confocal imaging of a nodule section incubated with anti-AOC. Images are shown with the color code shown in (B). Vacuolate cells (arrow) showed a stronger signal indicative for AOC than meristematic cells, which revealed only basal levels of immunofluorescence signal. (L) Bright-field semi-thin section showing cells of meristem and shoot bud primordia (SP). Bars in A, B, D, E, F, I, J, K, L = 100 µm; G, H = 50 µm; C= 25 µm.
Expression and jasmonate content in nodules taken 12 h upon induction was labeled. According to the color code displayed next to Fig. 5B red spots corresponding to maximum expression were observed in the cells (Fig. 5B arrows). Controls with the pre-immune serum displayed weak, non-specific autofluorescence (Fig. 5C).

Seven days after inoculation of internodes, cell divisions occurred in both cambial and cortical cells (Fig. 5D) and highly vacuolated callus cells appeared at the periphery. At this stage only few chloroplasts were observed (Fortes and Pais 2000). At day 15 prenodules were formed, which accumulated starch to a high level (Fortes and Pais 2000). An intense immunofluorescence signal indicative for AOC was found in amylloplasts of both proliferating cortical cells and prenodular cells (Fig. 5E).

At day 28 nodules were observed. Amyloplasts were still present in nodular cells though starch consumption had already started (Fortes and Pais 2000). AOC was still detectable in amylloplasts (Fig. 5G, 6A, B) and the cells of vascular bundles exhibited a remarkable amount of AOC (Fig. 5H).

Vacuolated cells of nodules also seem to contain AOC in amylloplasts and chloroplasts, which started to differentiate in some nodular cells at this stage. This was confirmed by staining with lugol (Fig. 6B) and co-localization with an anti-Rubisco antibody (Fig. 6D, arrows).

Upon 45 d after induction meristematic cells appearing at the periphery of the nodules displayed lower AOC immunofluorescence signal than vacuolated cells of nodules (arrow in Fig. 5J, K). Primordial cells of shoot buds showed a weak AOC signal in the cytoplasm (not shown). AOC was absent from the nucleus of nodular cells as revealed by double labeling with DAPI (Fig. 6C). In controls with a pre-immune serum only a weak autofluorescence signal was detected at the periphery (Fig. 5F). This signal probably corresponds to the cutin layer covering the ON (Fortes et al. 2002).

**Transient increase of JA and OPDA during nodule formation**

In order to quantify JA levels at different stages of ON formation HPLC separation and GC-MS analysis were used. Additionally, immunological detection was carried out by a competitive ELISA assay (Royo et al. 1999) providing similar results (not shown).

During ON induction, a 10-fold increase in the levels of JA was detected 4 h after internode inoculation followed by a decrease until 8 h (Fig. 7A). Interestingly, the OPDA level increased also transiently, but peaked at 12 h (Fig. 7B). The maximal value of OPDA was 15-fold higher than that of JA.

During ON development, the JA level massively decreased 7 d after induction and returned to a base level up to 45 d after inoculation (Fig. 8A). The OPDA level decreased with kinetics similar to those of JA (Fig. 8B). Again, the absolute amount of OPDA exceeded that of JA dramatically. Since the water content per g FW changed during development of internodes, prenodules, ONs and plantlets regenerating ONs due to significant callus formation the levels of JA and OPDA were also calculated in terms of milligrams of protein (Fig. 9A, B). We found that JA and OPDA increased two-fold and five-fold, respectively, during plantlet regeneration thus supporting previous observations.

**Discussion**

Jasmonates have been shown to play an important role in the response to abiotic and biotic stresses. Although less well studied than their influence on plant defense reactions,
Jasmonates are also known to be involved in several developmental processes: tuber formation, tendril coiling, seed germination, root growth, flower and fruit development and senescence (reviewed by Wasternack and Hause 2002, Wasternack 2004).

Here we show correlative data that suggest a putative role of jasmonates in organogenic nodule formation and plantlet development. Organogenic nodule formation is a morphogenic process suitable for use in regeneration strategies, automated micropropagation and genetic transformation for desirable characteristics (McCown et al. 1988). It plays a pivotal role in plant biotechnology together with somatic embryogenesis although little is known about the processes involved in their induction and development.

Organogenic nodule formation in hop and the involvement of wounding in its induction have been described elsewhere (Fortes and Pais 2000, Fortes et al. 2004, Silva et al. 2004). Culturing of long internodes (>10 mm) not submitted to incisions before inoculation impaired or led to a decreased yield in organogenic nodule formation (Fortes 2003, Fortes et al. 2004), suggesting that wounding may be involved in triggering the expression of morphogenetic competence.

AOS is regarded as the first enzyme specific for JA synthesis. The AOC acting subsequently, however, is of special importance since this enzyme establishes the naturally occurring enantiomeric form of jasmonates (Ziegler et al. 2000). In most plant species analyzed so far, AOS and preferably AOC play a regulatory role in JA biosynthesis, which is regulated by positive feedback dependent on substrate availability (reviewed by Wasternack and Hause 2002). In A. thaliana the preferential role of AOC for JA biosynthesis was shown by a comparison between the JA-deficient mutant opr3 affected in OPR3 with the corresponding wild type (Stenzel et al. 2003b).

In order to analyze JA biosynthesis during nodule formation in hop, the preferential role of AOC prompted us to clone a cDNA coding for AOC. The reverse transcriptase–PCR-based approach used here led to an ORF highly similar to two other published hop cDNAs and to the tomato AOC. A sequence similarity search revealed high homology of the putative AOC from hop with more than 35 AOC sequences analyzed previously (Stenzel et al. 2003b). This suggested that the protein detected here with a polyclonal antibody raised against recombinant tomato AOC is an AOC. With this antibody also AOCs of tobacco, Arabidopsis, M. truncatula and potato could be detected.

The AOC coding region encompasses 765 bp encoding a 255 amino acid protein with a calculated molecular mass of 28 kDa. Here we suggest that the difference of about 4 kDa
between the deduced molecular mass and that determined by immunoblotting is, in part, due to the post-translational removal of amino acids at the N-terminus as published earlier for tomato (Ziegler et al. 2000). Using the TargetP V1.0 program (Emanuelsson et al. 2000) we confirmed the presence of a chloroplast transit peptide at the N-terminus. This is in agreement with the immunocytochemical detection of AOC protein in chloroplasts (Fig. 6). All AOCs studied so far were found to be localized in the chloroplast (Feussner and Wasternack 2002). Based on these results we measured the enzyme activity of recombinant hop AOC expressed in E. coli. Mature AOC uses allene oxide as a substrate to convert it to cis-(+)-OPDA. Lower but significant enzyme activity was detected for the complete sequence. Hence, it may be that the chloroplast signal peptide does not fully inhibit the activity.

The role of AOC and JA/OPDA was studied during induction and development of ONs derived from hop internodes. Levels of JA at time zero were high (≈130 pmol (g FW)^{-1}) compared with leaf tissue of other plant species such as barley and tomato, which showed values at least 10 times lower (Kramell et al. 2000, Hause et al. 2000). In fact, the levels of JA, OPDA and other intermediates of oxylipin synthesis vary considerably among species, suggesting that the oxylipin signature may provide flexibility to this multifunctional chemical signaling system (Reymond et al. 2000).

Upon induction of ON the JA level increased about 10-fold within 4 h. Such an increase is known for leaf tissues of many plant species upon wounding. In wounded leaves, however, a transient rise usually appears within 1 or 2 h of wounding (e.g. tomato; Stenzel et al. 2003a).

Interestingly, OPDA accumulation peaked later than JA, and reached up to a 15-fold higher level following wounding. These data provoke the question on JA-independent signaling properties of OPDA. Indeed, OPDA-specific responses such as tendril coiling (Stelmach et al. 1998) and increased levels of JA and OPDA were observed during seedling development in rapeseed and flax (Wilen et al. 1991). Recently, increased levels of JA and OPDA were observed during seedling development in A. thaliana (Hause et al. 2003b). During plantlet regeneration from ON (after 45 d), there was an increase in both OPDA and JA levels occurred, probably due to lack of substrate generation, since AOC levels were still high. In contrast, during internode response to wounding AOC levels correlated with elevated levels of OPDA and JA. These two oxylipins showed identical kinetics during development of nodules and plantlet regeneration. During prenodule formation, the photosynthesis rate is reduced since most chloroplasts differentiate into amyloplasts, which accumulate starch to a large extent (Fortes and Pais 2000). A role of JA in the down-regulation of photosynthetic genes has been proposed and attributed to protection from oxidative stress in tissues lacking radical scavengers like chlorophyll (Creelman and Mullet 1997). It is thus possible that the high level of JA and OPDA observed 24 h after wounding of internodes may play a role in the down-regulation of photosynthetic genes during inhibition of chlorophyll synthesis and amyloplast differentiation observed in the early days following culture initiation (Fortes and Pais 2000). A correlation of high JA levels in photosynthetically inactive tissues was also observed in seedling development of barley (Hause et al. 1996, Maucher et al. 2000).

JA has been discussed to affect growth and differentiation processes by conditioning storage or mobilization of sugars. A well-studied example is the formation of tubers, where in response to JA, an increased level of sucrose followed by increased cell expansion were observed within the tuber-forming stolons (Takahashi et al. 1995, Cenzano et al. 2003). It is tempting to speculate that the high levels of JA and OPDA during induction of ON formation cause growth, cell expansion and cell differentiation as observed in other tissues and organs (Creelman and Mullet 1995, Koda 1997).

In nodules, AOC is preferentially located in cells close to vascular bundles (Fig. 5) suggesting tissue-specific generation of JA and OPDA. Such a local formation of JA was particularly found in wound tomato leaves (Stenzel et al. 2003a), which contain AOC protein exclusively in vascular bundles and sieve elements (Hause et al. 2003a). The preferential occurrence of AOC in vascular bundles accompanied with a local rise in JA may facilitate carbon partitioning which is known to be affected by JA (Creelman and Mullet 1997).

During plantlet regeneration from ON (after 45 d), there was an increase in both OPDA and JA levels. At this stage starch is being consumed (Fortes and Pais 2000) leading to elevated levels of glucose, which may induce AOC expression and increased JA levels (Hause et al. 2000). Jasmonic acid is regarded to be an endogenous regulator of embryo development in rapeseed and flax (Wilen et al. 1991). Recently, increased levels of JA and OPDA were observed during seedling development in A. thaliana (Hause et al. 2003b). During plantlet regeneration from ON the increase in OPDA levels
(five-fold) was higher than that detected for JA (two-fold) raising the question of JA-independent signaling properties of OPDA. In tomato flower organs different oxylipins signatures with high OPDA levels were found (Hause et al. 2000) and the constitutive overexpression of AOC led to extremely high levels of OPDA and OPDA methyl ester accompanied by down-regulation of 18:2-derived 9-LOX products (Miersch et al. 2004). These data also suggest the role of OPDA in distinct developmental stages. Thus, ON formation and plantlet regeneration as well as several morphogenic processes (Koda 1997) might be affected by JA and possibly even more by OPDA.

**Material and Methods**

**Plant material and culture conditions**

The internodes from *H. lupulus* (var. Nugget) plants, maintained under in vitro conditions, were morphologically induced according to the protocol described previously (Fortes and Pais 2000). Internodes of 6–9 mm long were wounded throughout by several incisions (3–5) using a razor blade (wounding treatment) before inoculation. Material corresponding to the following morphogenic stages was collected: stage zero corresponding to internodes at the time of excision from the parent plant; 2, 4, 8, 12 and 24 h from internode inoculation; 7 d after culture initiation corresponding to divisions in cambial and cortical cells of internodal explants; 15 d on culture medium in which several prenodular structures are formed inside the calluses; 28 d after culture initiation corresponding to nodule formation; 45 d on culture medium in which plantlet regeneration occurs from ONS.

**Cloning of AOC homolog from hop and RNA analyses**

Total RNA was isolated essentially as described by Rerie et al. (1991) from internodes following 0, 2, 4, 8 12 and 24 h after wounding and from material at various morphogenic stages. To further purify RNA treatment was carried out with DNase according to the suppliers’ instructions (Invitrogen, San Diego, CA, USA). Samples were then extracted in phenol/chloroform/isoamylalcohol (75 : 24 : 1, v/v/v), precipitated with sodium acetate and ethanol, washed in 70% ethanol and dissolved in water.

For cloning of hop AOC and AOS, a reverse transcriptase–PCR-based cloning approach was used. Degenerated primers were designed through alignment of known sequences available at GenBank. For amplification of a cDNA fragment coding for putative AOC the following primers were used: AOCFw 1 CGHGIAYMGGHRDAGYCCGCHTA and an oligo(dT) (annealing temperature of 57°C). 5’end of cDNA coding for putative AOC was obtained using the Marathon™ cDNA amplification Kit (Clontech, Palo Alto, CA, USA). For amplification of a cDNA fragment coding for putative AOS the following primers were used: AOSFw CTHGACGSMBADAGYTTC and AOSRev GTYTCSGHGCCRTHGACCA (annealing temperature of 53°C). The amplicons were cloned using the pGEM cloning kit (Promega, Madison, WI, USA).

Total RNA (20 μg per lane) was separated by 1% formaldehyde–agarose gel electrophoresis and blotted to Hybond-XL nylon membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). Equal loading of RNA was confirmed by ethidium bromide staining. Gene-specific probes were amplified by PCR from cDNAs with primer sequences deduced from the hop AOC ORF and the hop partial AOS. Hybridization was performed under high-stringency conditions at 60°C using probes labeled by Megaprime™ DNA labeling systems (Amersham Bioscience, Piscataway, NJ, USA). RNA gel-blot hybridizations were repeated twice from independent experiments.

**Expression of recombinant AOC in E. coli and AOC activity assay**

For expression in *E. coli*, the full-length AOC sequence and the truncated AOC sequence corresponding to 70–225, thus lacking the putative chloroplast target sequence, were both individually subcloned into pQE30 (Quiagen, Valencia, CA, USA). The pQE30 constructs were transformed into the host strain *E. coli* M15. Total protein of isopropyl-β-thio-galactopyranoside ( IPTG)-induced or non-induced cultures was isolated and purified as described previously (Maucher et al. 2000).

Total extracts from independent cultures were used for activity tests as described by Dorer (2004). 135-hydroxyprop-9(Z, 11E, 15Z)-octadecatrienoic acid was converted by the auxiliary enzyme AOS from barley to form 12,13-epoxylinolenic acid. This unstable allene oxide is transformed by AOC to cis-(+)-OPDA (9S, 13S). The reaction was carried out for 5 min on ice in 100 mM phosphate buffer, pH 7.0 and 10 mM 13-HPOT. The reaction was stopped with HCl and the OPDA was extracted. As 12,13-epoxylinolenic acid can spontaneously degrade to α-ketol, γ-ketol and both chiral isomers of OPDA, the extracted products were separated in two separate HPLC steps. After NaOH transformation the first HPLC separated the ketols from both OPDA isomers on an isocratic gradient run on a RP-18 column. The fraction of the OPDA peak was then separated on a chiral phase HPLC column (Nucleosil β-PM, Macherey-Nagel, Düren, Germany). The isomer (9R,13R)-(−)-OPDA arises both from the AOC enzyme reaction and from spontaneous cleavage; whereas (9R,13S)-(−)-OPDA arises only from the degradation reaction. Because 12,13-epoxylinolenic acid degrades equally to both chiral isomers, a shifted ratio towards the (9R,13R)-(−)-OPDA isomer was indicative for AOC enzyme activity. Thus the activity was calculated as the (shifted) ratio of (9R,13S)-(−)-OPDA to (9S,13R)(−)-OPDA.

**Extraction of proteins and immunoblot analysis**

Proteins were extracted as described previously (Fortes et al. 2004) using an extraction buffer as described by Borrel et al. (1997). Proteins were subjected to electrophoresis on a discontinuous gradient of SDS–polyacrylamide consisting of a 12% (w/v) acrylamide resolving gel and a 6% (w/v) stacking gel. Thirty micrograms of total protein was loaded per lane together with prestained standard proteins.

After electrophoresis, proteins were transferred to Immobilon membranes (Millipore; Bedford, MA, USA) and an equal loading was confirmed by Ponceau S staining. For immunodetection, membranes isolated and purified as described previously (Maucher et al. 2000). β-Actin (–)OPDA to (9R,13S)-(−)-OPDA, the fraction of the OPDA peak was then separated on a chiral phase HPLC column (Nucleosil β-PM, Macherey-Nagel, Düren, Germany). The isomer (9R,13R)-(−)-OPDA arises both from the AOC enzyme reaction and from spontaneous cleavage; whereas (9R,13S)-(−)-OPDA arises only from the degradation reaction. Because 12,13-epoxylinolenic acid degrades equally to both chiral isomers, a shifted ratio towards the (9S,13R)-(+)OPDA isomer was indicative for AOC enzyme activity. Thus the activity was calculated as the (shifted) ratio of (9R,13S)-(−)-OPDA to (9S,13R)(+)OPDA.

**Histological analysis**

Samples were fixed overnight at 4°C in 4% (w/v) paraformaldehyde in PBS, pH 7.3. After washes in PBS, they were dehydrated in a methanol series at 4°C. Then, samples were washed in pure methanol, infiltrated and embedded in Lowicryl K4M at –30°C and polymerized under ultraviolet irradiation. Semi-thin sections were performed for preliminary histological analysis.

**Immunofluorescence assays**

Plant material at different time points was fixed overnight at 4°C in 4% (w/v) paraformaldehyde in PBS and then cryoprotected, embedded and sectioned as mentioned (Fortes et al. 2004). Immunolocaliza-
tion was performed essentially as described by Testillano et al. (1995) with minor modifications (Fortes et al. 2004). Cryostat sections were treated with 2% cellulse (Onozuka R-10) in PBS, blocked with 5% BSA and incubated for 1 h with a polyclonal antibody raised against AOC from tomato (Ziegler et al. 2000) diluted 1 : 50. Sections were further incubated with anti-rabbit Alexa Fluor 488 secondary antibody (Molecular Probes, Leiden, Netherlands) diluted 5 : 25 in Evans Blue (Sigma-Aldrich, St. Louis, MO, USA) to avoid non-specific auto-fluorescence of the cell walls. Alternatively, sections were further stained with DAPI or with lugol solution. Confocal optical sections were collected using a Bio-Rad MRC-1024 confocal scanning head mounted on a Zeiss Axiolab 135 microscope. Images of immuno-fluorescence assays were also collected using a cooled CCD camera (V-Scan Photonic Science) mounted on an Olympus IX-50 (Labocontrol). Images of sections stained with lugol were recorded using visible light in bright-field conditions. Controls were performed by replacing the first antibody with pre-immune serum.

For double immunofluorescence analysis sections were first incubated for 1 h with anti-Rubisco polyclonal antibody raised against the large subunit (generously offered by Dr R.T. Besford from Horticultural Research International, Little Hampton, West Sussex, UK) diluted 1 : 50. Assays were also performed with anti-AOC as the first antibody. Sections were incubated for 1 h with anti-rabbit Alexa Fluor 546 secondary antibody (Molecular Probes) diluted 5 : 25 in Evans Blue. Then, sections were incubated in 1% glutaraldehyde (Sigma-Aldrich) in PBS for 15 min and in 50 mM NH₄Cl in PBS (2×20 min each). Sections were further incubated with anti-AOC diluted 1 : 25. Incubation with anti-rabbit Alexa Fluor 488 secondary antibody diluted 1 : 25 was performed for 1 h followed by washes in PBS and incubation with 1% glutaraldehyde for 5 min. Sections were mounted in Moviol (Sigma-Aldrich). Confocal optical sections were collected as mentioned above. Merged images of both Rubisco and AOC immunofluorescence signals were recorded.

Quantification of JA and OPDA

Samples were collected at 0, 4, 8, 12 and 24 h and at 7, 15, 28 and 45 d after culture initiation, frozen in liquid nitrogen and kept at −80°C until analysis. For each sample 1 g of plant tissue was homogenized in a mortar and extracted in a mixture containing 10 ml methanol, 100 µg of [³H]JA and 100 ng of [³H]OPDA as internal standards. Extractions were carried out under constant stirring at 4°C overnight. Purification and separation by HPLC and quantification by GC-MS were performed as described by Kramell et al. (2000). At least three independent replicates were measured for each time point.

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References


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