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The AOC promoter of tomato is regulated by developmental and environmental stimuli

Irene Stenzel [a,2,3], Bettina Hause [b,3], Reinhard Proels [c], Otto Miersch [a], Mariko Oka [a,1], Thomas Roitsch [c], Claus Wasternack [a,*]

[a] Leibniz Institute of Plant Biochemistry, Department of Natural Product Biotechnology, Weinberg 3, D-06120 Halle (Saale), Germany
[b] Leibniz Institute of Plant Biochemistry, Department of Secondary Metabolism, Weinberg 3, D-06120 Halle (Saale), Germany
[c] Julius-von-Sachs Institute of Biosciences, University of Wuerzburg, Julius-von-Sachs-Platz 2, D-97082 Wuerzburg, Germany

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ABSTRACT

The allene oxide cyclase (AOC) catalyzes the formation of *cis*-(+)-12-oxophytodienoic acid, an intermediate in jasmonate biosynthesis and is encoded by a single copy gene in tomato. The full length AOC promoter isolated by genome walk contains 3600 bp. Transgenic tomato lines carrying a 1000 bp promoter fragment and the full length promoter, respectively, in front of the β-glucuronidase (GUS)-encoding *uidA* gene and several tobacco lines carrying the full length tomato AOC promoter before *GUS* were used to record organ- and tissue-specific promoter activities during development and in response to various stimuli. High promoter activities corresponding to immunocytochemically detected occurrence of the AOC protein were found in seeds and young seedlings and were confined to the root tip, hypocotyl and cotyledons of 3-d-old seedlings. In 10-d-old seedlings promoter activity appeared preferentially in the elongation zone. Fully developed tomato leaves were free of AOC promoter activity, but showed high activity upon wounding locally and systemically or upon treatment with JA, systemin or glucose. Tomato flowers showed high AOC promoter activities in ovules, sepals, anthers and pollen. Most of the promoter activity patterns found in tomato with the 1000 bp promoter fragment were also detected with the full length tomato AOC promoter in tobacco during development or in response to various stimuli. The data support a spatial and temporal regulation of JA biosynthesis during development and in response to environmental stimuli.

1. Introduction

Jasmonates are signals in plant stress responses and development (for review see Wasternack, 2007). This group of compounds encompasses jasmonic acid (JA), its methyl ester (JAME), its amino acid conjugates and other metabolites such as 12-OH-JA. The octadecanoid *cis*-(+)-12-oxophytodienoic acid (OPDA) is the precursor of JA.

Initially, developmental programs such as root growth and senescence were found to be inhibited and promoted, respectively, by JA (Parthier, 1990), whereas tuber formation is induced by 12-OH-JA (Koda, 1992). Later on, Arabidopsis mutants with defects in JA biosynthesis and JA signaling were isolated and many of them are affected in pollen development (for review see Creelman and Mulpuri, 2002; Turner et al., 2002; Balbi and Devoto, 2008). One of these mutants is the male sterile, JA-insensitive mutant *coi1*. *COI1* codes for an F-box protein (Xie et al., 1998). In contrast, *jai1* plants, mutated in the tomato homolog of COI1, are female sterile (Li et al., 2004). Most recently, COI1 was shown to interact with JAZ proteins in the presence of JA isoleucine conjugate thereby indicat-

Abbreviations: α-LeA, α-linolenic acid; ACX1A, acyl CoA oxidase 1A; AOC, allene oxide cyclase; AOS, allene oxide synthase; COI1, F-box protein affected in the coronatine insensitive mutant *coi1*; DAG, day after germination; ET, ethylene; 13-HPOT, 13*S*-hydroperoxy(9*Z*,11*E*,15*Z*)-octadecatrienoic acid; GUS, β-glucuronidase; 12-HSO_4-JA, 12-hydroxyjasmonic acid sulfate; IAA, indolyl acetic acid; *jai*, JA-insensitive mutant; JA, jasmonic acid; JAME, JA methyl ester; JAZ, JA-ZIM-domain proteins; LAP, leucine amino peptidase; LOX, lipoxygenase; 12-*O*-Glc-JA, 12-hydroxyjasmonic acid glucoside; 12-OH-JA, 12-hydroxyjasmonic acid; OPDA, *cis*-(+)-12-oxophytodienoic acid; OPR, OPDA reductase; PBS, phosphate buffered saline; PCIB, 2-(4-chlorophenoxy)-2-methylpropionic acid; PIN, proteinase inhibitor; SA, salicylic acid; TD, threonine deaminase.

* Corresponding author. Tel.: +49 345 5582 1210; fax: +49 345 5582 1219.
E-mail address: cwastern@ipb-halle.de (C. Wasternack).
[1] Present address: Faculty of Agriculture, Tottori University, Koyamacho-minami 101, Tottori 680-8553, Japan.
[2] Present address: Albrecht-von-Haller-Institute for Plant Sciences, Georg-August University of Goettingen, Department of Plant Biochemistry, Justus-von-Liebig-Weg 11, D-37077 Goettingen, Germany.
[3] The first two authors contributed equally to this work.

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ing putative receptor function of the complex (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007).

In the last decade, JA and OPDA were recognized as partially independent signals, which act in responses to biotic and abiotic stress such as herbivore or pathogen attack and desiccation, respectively (reviewed in Wasternack, 2007; Howe and Jander, 2007). Upon such an environmental stimulus, endogenous rise of JA and OPDA caused by *de novo* biosynthesis occur and is part of a complex signal transduction pathway (Balbi and Devoto, 2008).

JA biosynthesis is one of seven different branches of the lipoxygenase (LOX) pathway (Feussner and Wasternack, 2002), where an initial LOX-catalyzed oxygenation of α-linolenic acid (α-LeA) or linoleic acid is the initial reaction. The oxygen can be inserted at position 9 by a 9-LOX or at position 13 by 13-LOX. The latter reaction leads to 13*S*-hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic acid (13-HPOT), the substrate for a 13-allene oxide synthase (13-AOS). The unstable allene oxide is converted by an allene oxide cyclase (AOC), which catalyzes the formation of *cis*-(+)-OPDA (9*S*,13*S*). This compound carries the enantiomeric structure of the naturally occurring JA. Consequently, AOC is of special importance in regulation of JA biosynthesis (for review cf. Wasternack, 2006).

The cyclopentenone OPDA is reduced by an OPDA reductase (OPR) and β-oxidized in the carboxylic acid side chain. Most of the genes encoding enzymes of JA biosynthesis have been cloned from several plant species. For tomato, a cDNA coding for a 13-LOX (LOXD) has been identified to function in JA formation (Heitz et al., 1997). Furthermore, two 13-AOSs (Sivasankar et al., 2000; Howe et al., 2000), one AOC (Ziegler et al., 2000) and three OPRs with role of OPR3 in JA biosynthesis (Strassner et al., 2002) have been cloned. In case of β-oxidation of the carboxylic acid side chain of OPDA, a tomato acyl CoA oxidase (ACX1A) has been cloned, and its role in JA formation was shown (Li et al., 2005). Transgenic approaches with *antisense*-mediated suppression of genes encoding enzymes in fatty acid β-oxidation supported these data (Cruz Castillo et al., 2004). Expression analyses including microarray analyses revealed for most of JA biosynthetic genes a COI1-dependent activation upon wounding, pathogen attack or jasmonate treatment (Reymond et al., 2000, 2004; Devoto et al., 2005). This latter fact confirms the positive feedback loop in JA biosynthesis, which was repeatedly shown including transgenic approaches (Wang et al., 1999; Laudert et al., 2000; Stenzel et al., 2003; Strassner et al., 2002) (for details cf. review in Wasternack et al., 2006).

Organ- and tissue-specific expression of tomato genes encoding JA biosynthetic enzymes is much less understood. In case of *LOXD*, expression was determined for leaves and different flower organs (Heitz et al., 1997). The *9-AOS* is specifically expressed in roots (Itoh et al., 2002), whereas the *13-AOSs* are expressed in stems and less in roots and flowers (Howe et al., 2000). The AOC protein encoded by a single copy gene (Ziegler et al., 2000) occurs preferentially in vascular tissues and in ovules of flower buds and open flowers (Hause et al., 2000; Stenzel et al., 2003; Miersch et al., 2004). A detailed inspection revealed the occurrence of tomato AOC in companion cells and sieve elements of vascular bundles (Hause et al., 2003). The tomato OPR3 mRNA was detected in roots, leaves and flowers (Strassner et al., 2002). The tomato *ACX1A* is expressed in leaves but organ-specific expression was not analyzed until now. Furthermore, a detailed analysis of tissue-specific expression including different developmental stages and promoter activity data is still missing for all tomato genes encoding JA biosynthetic enzymes. Due to the preferential role of the single copy gene AOC for regulation of JA formation, its promoter activities are of special interest. This prompted us to analyze comparatively *SlAOC* promoter activities using tomato and tobacco plants. Here, we show isolation of the tomato AOC promoter and its activity analysis in transgenic lines of tomato and tobacco transformed with chimeric *SlAOC* promoter-β-glucuronidase (GUS) fusions. Reporter gene activities were recorded histochemically during development of different organs and under various treatments known to increase JA levels and were compared to AOC expression data and JA levels. The data suggest spatial and temporal regulation of AOC promoter activities during development and in response to environmental cues.

2. Results

2.1. Cloning and sequence analyses of the AOC promoter

In order to isolate the 5′-sequences responsible for regulating expression of AOC, a 5′-genome walk was performed on genomic libraries of tomato starting from exon I of AOC. A single walk yielded a 3600 bp fragment of 5′-sequences comprising the full length AOC promoter.

Sequence analyses of the AOC promoter sequence revealed a putative transcription start site 68 bp 5′ of the translation start codon as deduced by comparison with reported consensus sequences (Joshi, 1987) and the AOC mRNA sequence (Acc. Nr. AF384374). A putative TATA-box (TATATAA) appears at −34 (5′ of the putative transcription start site) and a putative CAAT-box at position -79. In addition, the promoter sequence was analyzed for the presence of *cis*-acting elements. *cis*-Acting elements involved in the transcriptional regulation of JA/ET/SA-dependent responses were analyzed by Adie et al. (2007) and yielded several over-represented sequences in corresponding promoters. Among those, the GCC-box, G-box, and W-box were found to be predominant elements. Analyzing the AOC promoter, we identified those sequences at position – 635 (GCC-box, AGCCGCA, base pair exchanges compared to consensus sequence are underlined) and −3267 (GCC-box, CGCCGCC), −589 (G-box, CACGTG) and −2811(W-box, TTGACC), −2416 (W-box, reverse orientation) and +38 (W-box, TTGACC). Furthermore, an elicitor-response element of an *Arabidopsis* PAL promoter, as described by Ohl et al. (1990), was found at position +49 (AACCAACAA) on the AOC promoter.

To analyze of the AOC promoter function in planta *via* GUS activity, fragments containing 1000 and 3600 bp, respectively, of promoter sequences were fused to the β-glucuronidase gene, and transgenic tomato and tobacco plants expressing the corresponding reporter gene fusions were generated.

2.2. Tomato AOC (1000 bp) promoter activities during development of seedlings, flowers and fruits of tomato

Already before imbibition, transgenic seeds showed AOC promoter activity (Fig. 1A), which was equally distributed and was kept one day after germination (DAG) and two DAGs (Fig. 1B and C). After three DAGs, preferential promoter activity appeared at the primary root tip and the hypocotyl (Fig. 1D). In cross-sections of 3-d-old cotyledons, strong AOC protein accumulation is visualized by immuno-staining (Fig. 1E–G). The DIC image of a longitudinal section indicates that the AOC promoter activity is localized in meristematic tissue of the primary root (Fig. 1I), and cross-sections at different regions of this tissue show activity in the epidermal layer and the meristematic cells (Fig. 1H). As visualized by immuno-staining of longitudinal sections of a primary root, the AOC protein is mainly located in cells showing AOC promoter activity (Fig. 1J versus I). In 10-d-old seedlings AOC promoter activity was visible mainly in the main vein. Other tissues with promoter activity are the tip of the main root, the hypocotyl as well as the stalk and the tip of the primary leaves (Fig. 1K). Higher magnification of the main root tip shows, compared to the 3-d-old seedling, a shift of AOC promoter activity into the elongation zone (Fig. 1L).

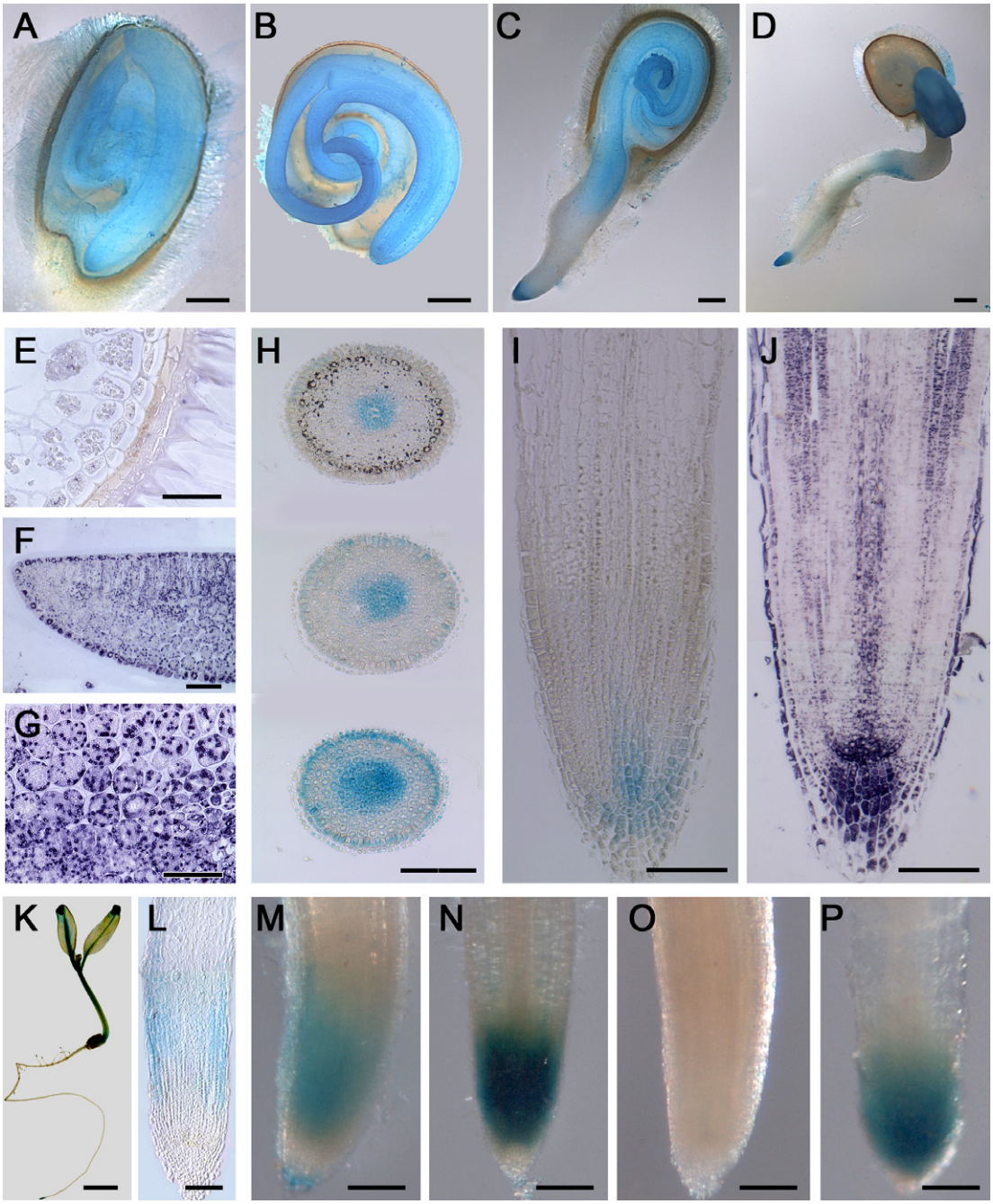

Fig. 1. AOC (1000 bp) promoter activity and AOC immunolocalization in seedling development of tomato. (A) Dry seeds; (B) 1 DAG; (C) 2 DAG; (D) 3 DAG; (bars in A–D: 500 μm), (E) immuno-staining of cross-section of seed coat and endosperm and (F and G) of cross-section of cotyledons of a 3-d-old seedling (bars: 50 μm), (H) cross-sections of meristematic tissues of the root tip of a 3-d-old seedling shown in I (bar: 100 μm); (I) primary root of a 3-d-old seedling (DIC image, bar: 100 μm); (J) immuno-staining with an anti-AOC-antibody of a primary root of a 3-d-old seedling (bar: 100 μm); (K) 10-d-old seedling; (L) primary root of a 10-d-old seedling (DIC image), (M) primary root of a 7-d-old seedling; (N) as in (M), but treated with 10 μM JAME for 24 h; (O) as in (M), but treated with 100 μM IAA for 24 h and (P) as in (M) but treated with 100 μM PCIB for 24 h (bars for L–P: 100 μm).

AOC promoter activity in the primary root of a 7-d-old seedling is increased by JAME treatment (Fig. 1N versus M), but is inhibited by IAA treatment (Fig. 1O). The IAA effect can be compromised by the IAA-inhibitor PCIB (Fig. 1P).

The preferential AOC promoter activity and occurrence of the AOC protein in the root tip suggest a higher accumulation of JA, OPDA and JA-metabolites in the root tip than in the root. Quantitative analysis of root tips and roots of 7-d-old seedlings in respect to the content of JA, OPDA, 12-OH-JA, 12-*O*-Glc-JA and 12-HSO_4-JA indicated for each compound higher levels in the root tip (Table 1).

Table 1
Content of JA, OPDA, 12-OH-JA, 12-*O*-Glc-JA and 12-HSO_4-JA roots and root tips of 7-d-old seedlings (data in pmol per g f.w.) (±SD from three biological replicates and three technical replicates is given)

	JA	OPDA	12-OH-JA	12-*O*-Glc-JA	12-HSO_4-JA
Root	450 ± 45	450 ± 42	100 ± 12	200 ± 21	1420 ± 150
Root tip	1980 ± 190	1050 ± 110	150 ± 16	250 ± 24	1850 ± 190

In flowers before opening, high AOC promoter activities appeared in sepals and the pistil (Fig. 2A). In a cross-section of an 8-mm flower bud, mainly ovules exhibited AOC promoter

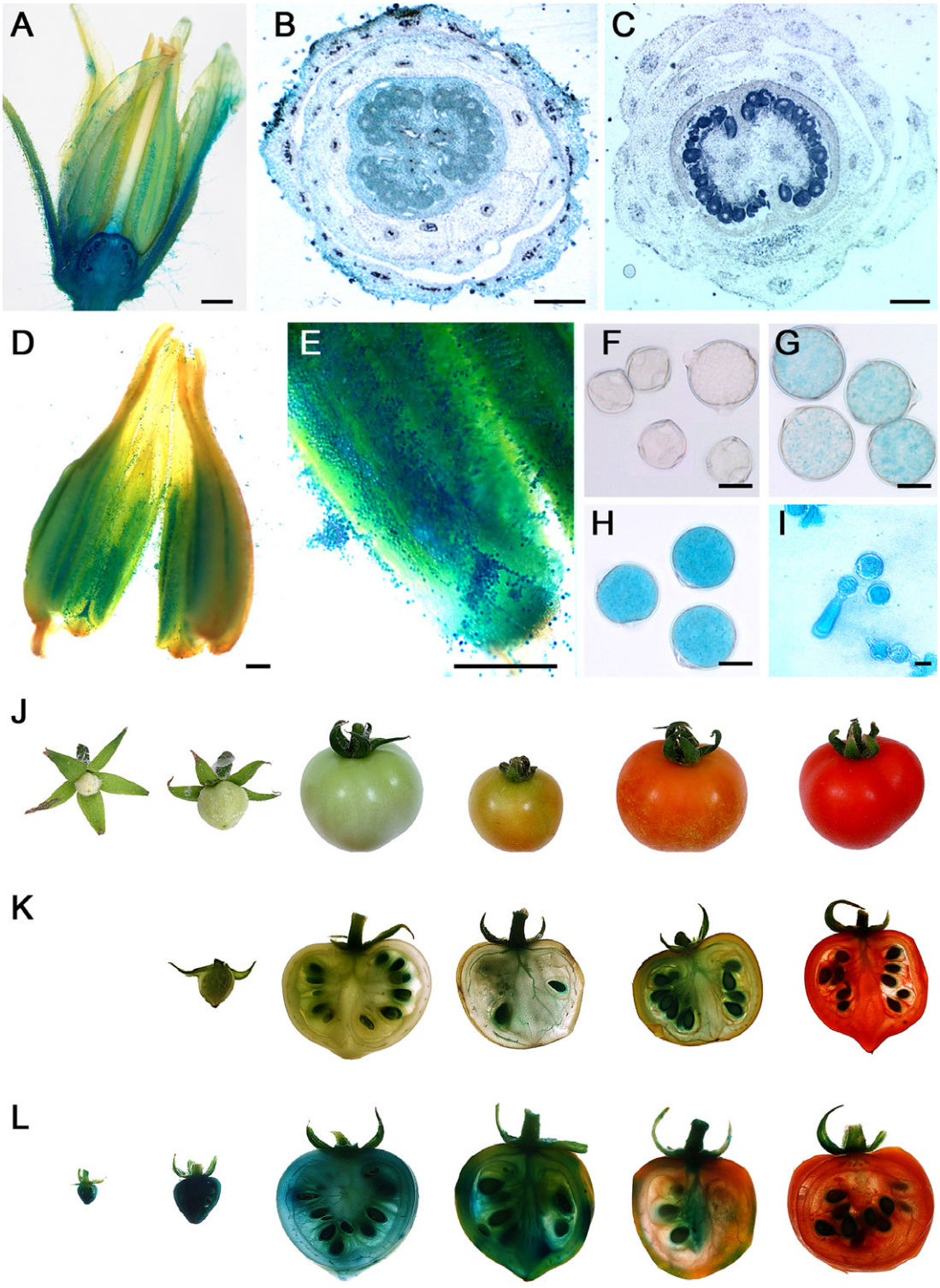

Fig. 2. AOC (1000 bp) promoter activity and AOC immunolocalization in flower and fruit development of tomato. (A) Flower before anthesis; (B) cross-section of a flower bud; (C) immuno-staining with an anti-AOC antibody in a cross-section of a flower bud; (D) anthers; (E) higher magnification of an anther (bars in B–E: 500 μm); (F) early bicellular pollen; (G) middle bicellular pollen; (H) late bicellular pollen; (I) germinating pollen (bars in F–I: 10 μm; (J and K) fruit development of wt microtom plants; and (L) AOC (1000 bp) promoter activity during fruit development.

activity (Fig. 2B), which corresponds to location of AOC protein visualized by immuno-staining (Fig. 2C). In contrast, in fully open flowers AOC promoter activity is detectable in anthers and the released mature pollen (Fig. 2D and E). A more detailed inspection of pollen development shows an increase in AOC promoter activities between the early and the late bicellular pollen (Fig. 2F–H) and activity in the germinating pollen (Fig. 2I).

During fruit development, an AOC promoter activity was found preferentially in seeds, but decreasing activity appeared in fruit tissue (Fig. 2L, for comparison cross-sections (Fig. 2K) and whole fruits of the wt microtom (Fig. 2J) are shown).

2.3. Tomato AOC (1000 bp) promoter activity in tomato leaves and in response to wounding and to other treatments

Untreated young leaves exhibit AOC promoter activity preferentially in the vascular tissue (Fig. 3A). In fully developed leaves (Fig. 3B) and later stages of leaf development including senescent leaves, AOC promoter activity was not detectable (data not shown). Two hours upon wounding of detached leaflets, AOC promoter activity appeared preferentially in vascular tissue, but was distributed all over the leaf after 18 h (Fig. 3C). The preferential AOC promoter activity in vascular tissue is also documented in cross-sections of the petiole (Fig. 3E versus D).

Treatments of leaves with compounds known to be signals in the wound response, such as JA, systemin or glucose indicate increased AOC promoter activity for all of them. Treatment with 50 μM JA (Fig. 3G), 100 pM systemin (Fig. 3H) and 0.5 M glucose (Fig. 3I) for 2 h led to preferential AOC promoter activities in the vascular tissue in comparison to the untreated control (Fig. 3F). Similar results were found with the full length (3600 bp) AOC promoter GUS lines of tomato and tobacco (data not shown).

The preferential AOC promoter activity in vascular tissue is visualized in more detail by cross-sections near the wound-side (Fig. 3J–L), which is schematically shown in Fig. 3M. Beside residual AOC promoter activity in vascular bundles of an untreated leaf, activity increased upon treatment with 100 pM systemin, but was confined to vascular bundles (Fig. 3K). In contrast, upon wounding for 2 h AOC promoter activity appeared in vascular bundles and the surrounding mesophyll cells (Fig. 3L). Eighteen hours after wounding AOC promoter activity appeared in all leaf tissues (data not shown), thus corresponding to data of Fig. 3C. Local wounding led to AOC promoter activity at the wound-side preferentially in vascular tissue after 2 h (Fig. 4B versus A), but was progressively distributed to systemic leaves 8 h after wounding (Fig. 4C). Transgenic tomato plants expressing the AOC (3600 bp) promoter GUS fusion were used to quantify the induction by glucose and jasmonate. Both 500 mM glucose and 50 μM JA strongly increased the basic level of AOC promoter activity (200 nmol/min × μg protein). The magnitude of induction varied between the different transgenic lines and the promoter activity was 400–600% higher in response to 500 mM glucose and 200–400% higher in response to 50 μM JA, whereas 50 mM glucose did not significantly affect the promoter activity.

To inspect whether the distribution of increased AOC promoter activity upon wounding corresponds to movement of JA in this

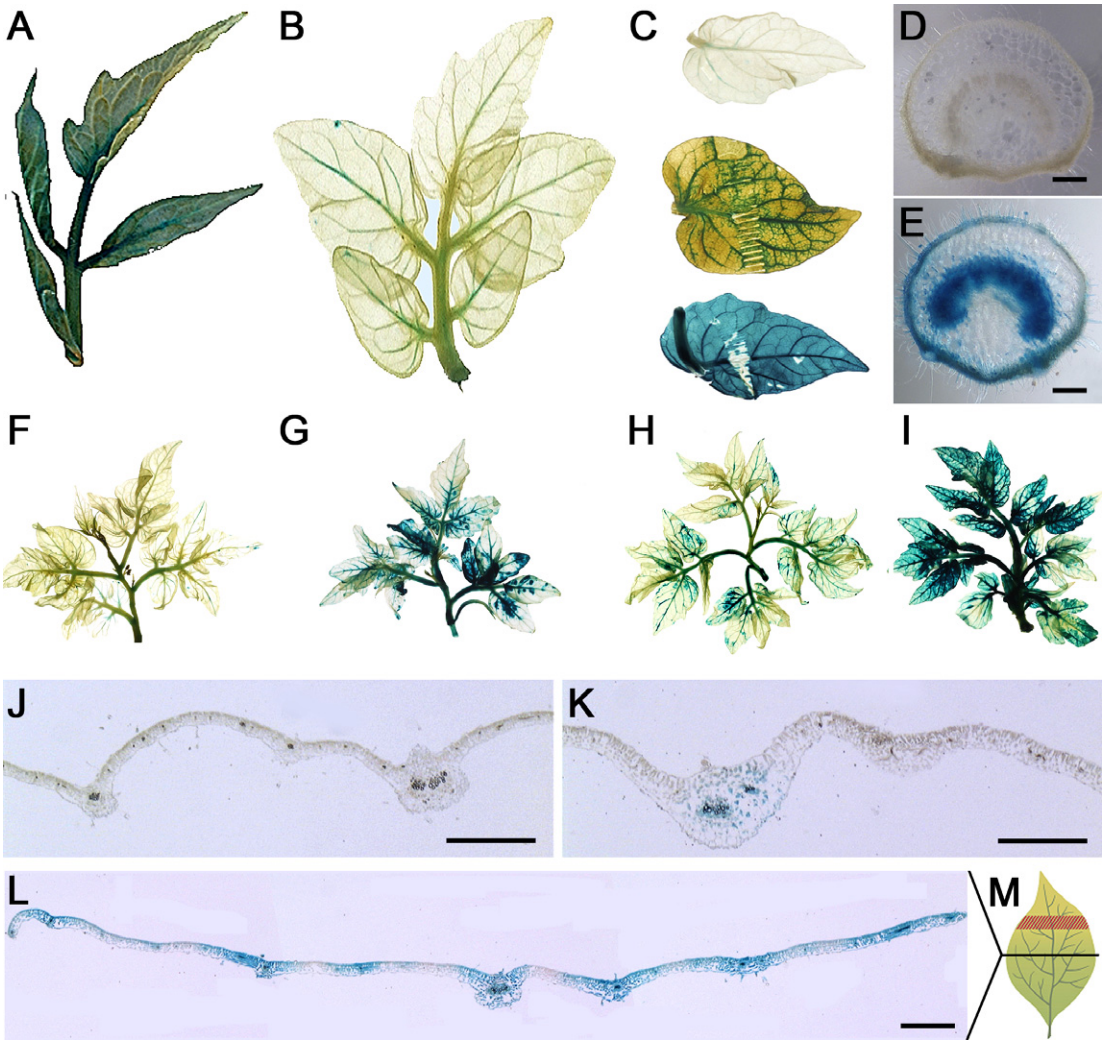

Fig. 3. AOC (1000 bp) promoter activity in leaves upon wounding and other treatments. (A) Young leaf; (B) green leaf; (C) fully developed leaflets, untreated (top), 2 h after wounding (middle) and 18 h after wounding; (D) cross-section of an untreated petiole; (E) cross-section of a petiole 2 h after wounding of the leaflet; (F) untreated leaf; (G) leaf treated with 50 μM JA for 2 h; (H) leaf treated with 100 pM systemin for 2 h; (I) leaf treated with 0.5 M glucose for 2 h; (J) cross-section of an untreated leaflet; (K) cross-section of a leaflet treated with 100 pM systemin for 2 h; (L) cross-section of a leaflet 2 h after wounding (bars in J–L: 100 μm) and (M) scheme for wounding and position of the cross-section.

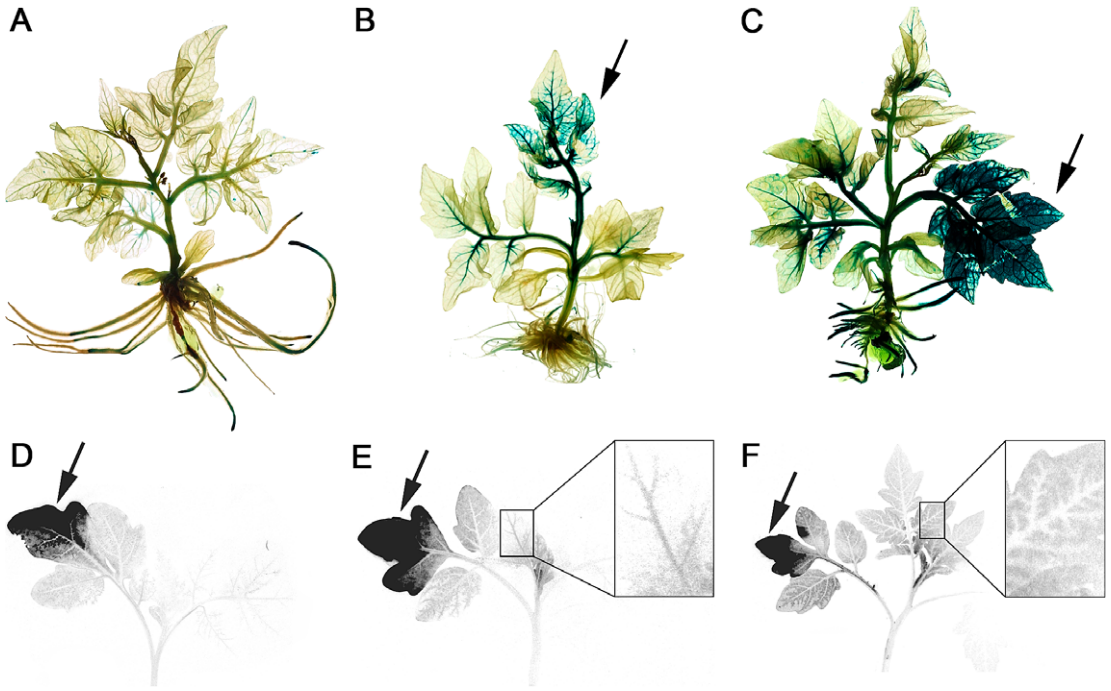

Fig. 4. Local and systemic AOC (1000 bp) promoter activity and autoradiography of a wt MicroTom plant fed with ^{14}C-labeled JA. (A) Untreated; (B) 2 h after wounding, (C) 8 h after wounding; (D)–(F) Autoradiographs showing distribution of ^{14}C-labeled JA 0 h, 3 h and 24 h, respectively, after feeding. Insets in (E) and (F) show higher magnification of the marked area. Arrows indicate the site of wounding (B, C) or the site of injection (D–F).

time frame, we injected ^{14}C-labeled JA locally and inspected its distribution at different times after injection by autoradiography (Fig. 4D–F). Whereas 3 h after injection radioactivity appeared preferentially in vascular bundles of the systemic leaf (Fig. 4 inset in E), thus corresponding to promoter activity (Fig. 4B), 24 h after injection radioactivity appeared in the intercostal regions of the leaf (Fig. 4 inset in F). Most of the radioactivity was due to ^{14}C-JA; whereas the remaining portion consisted of a mixture of acidic JA metabolites (Bücking et al., 2004).

2.4. Tomato AOC (3600 bp) promoter is active in tobacco flowers

For comparison with a higher number of transgenic lines, *Nicotiana tabacum* (cv. SN) plants were transformed with a construct carrying the full length tomato AOC promoter and the GUS reporter gene.

In young flower buds, 12 mm in size, full length promoter activities could be detected in the pistil, in petals and sepals (Fig. 5A). At later stages, e.g. 25 mm buds and open flowers, AOC promoter activities appeared in ovules, sepals and also in anthers of open flowers (Fig. 5B and C). AOC promoter activities could not be detected in anthers before flower opening and not in filaments as well as the stigma of open flowers (Fig. 5B). Cross-sections of a 12 mm bud (Fig. 5G) and open flowers (Fig. 5H and I) illustrate preferential occurrence of AOC promoter activity in ovules and the integument. Corresponding to AOC promoter activity of ovules of an open flower (Fig. 5G–I), AOC protein could be detected in cross-sections by immuno-staining (Fig. 5D–F) with a residual staining by the preimmune serum (Fig. 5D′–F′).

3. Discussion

Jasmonates are central signals in plant stress responses and development. In the well-studied wound response of tomato, JA is generated systemin-dependently (Stenzel et al., 2003). Sequentially acting processes such as *PROSYSTEMIN* expression → systemin formation → *AOC* expression → JA formation → prosystemin expression may attribute to amplification in wound signaling (Ryan, 2000; Stenzel et al., 2003; Schilmiller and Howe, 2005). This is supported by a common location of *PROSYSTEMIN* expression and AOC expression in vascular tissues (Jacinto et al., 1997; Narváez-Vásquez and Ryan, 2004; Hause et al., 2000), and in case of LOX, AOS and AOC proteins even in sieve elements (Hause et al., 2003). Such local amplification in wound-signaling may attribute to systemic role of JA or JA-derived compounds as suggested by grafting experiments with tomato mutants affected in JA biosynthesis and signaling (Schilmiller and Howe, 2005; Li et al., 2005; Howe and Jander, 2007). In this scenario, however, cell- and tissue-specific analysis of promoter activities for JA biosynthesis genes is missing.

3.1. AOC promoter activity upon wounding

Systemin-dependent AOC expression and JA formation upon wounding previously described (Stenzel et al., 2003; Wasternack et al., 2006) is substantiated here by AOC promoter activity data. Most of the data were collected with tomato lines carrying the AOC(1000 bp)-promoter, but nearly identical results were found with tomato lines carrying the full length promoter as well as with tobacco lines carrying the tomato AOC(1000 bp)-promoter or the tomato AOC(3600 bp)-promoter (cf. below and data not shown). Upon wounding, AOC(1000 bp)-promoter is rapidly activated in main veins of leaflets and petioles of tomato (Fig. 3C), thus corresponding well to the occurrence of AOC protein in these tissues (Hause et al., 2000), to the *PROSYSTEMIN* promoter activity (Jacinto et al., 1997) as well as to location of the prosystemin peptide (Narváez-Vásquez and Ryan, 2004). After 18 h, AOC(1000 bp)-promoter activity is detectable also in mesophyll cells (Fig. 3C and L), where the most prominent wound-responsive *PROTEINASE INHIBITOR2* (*PIN2*) gene is expressed (Schilmiller and Howe, 2005). Wound-responsive genes were grouped into early genes such as *LOX* and

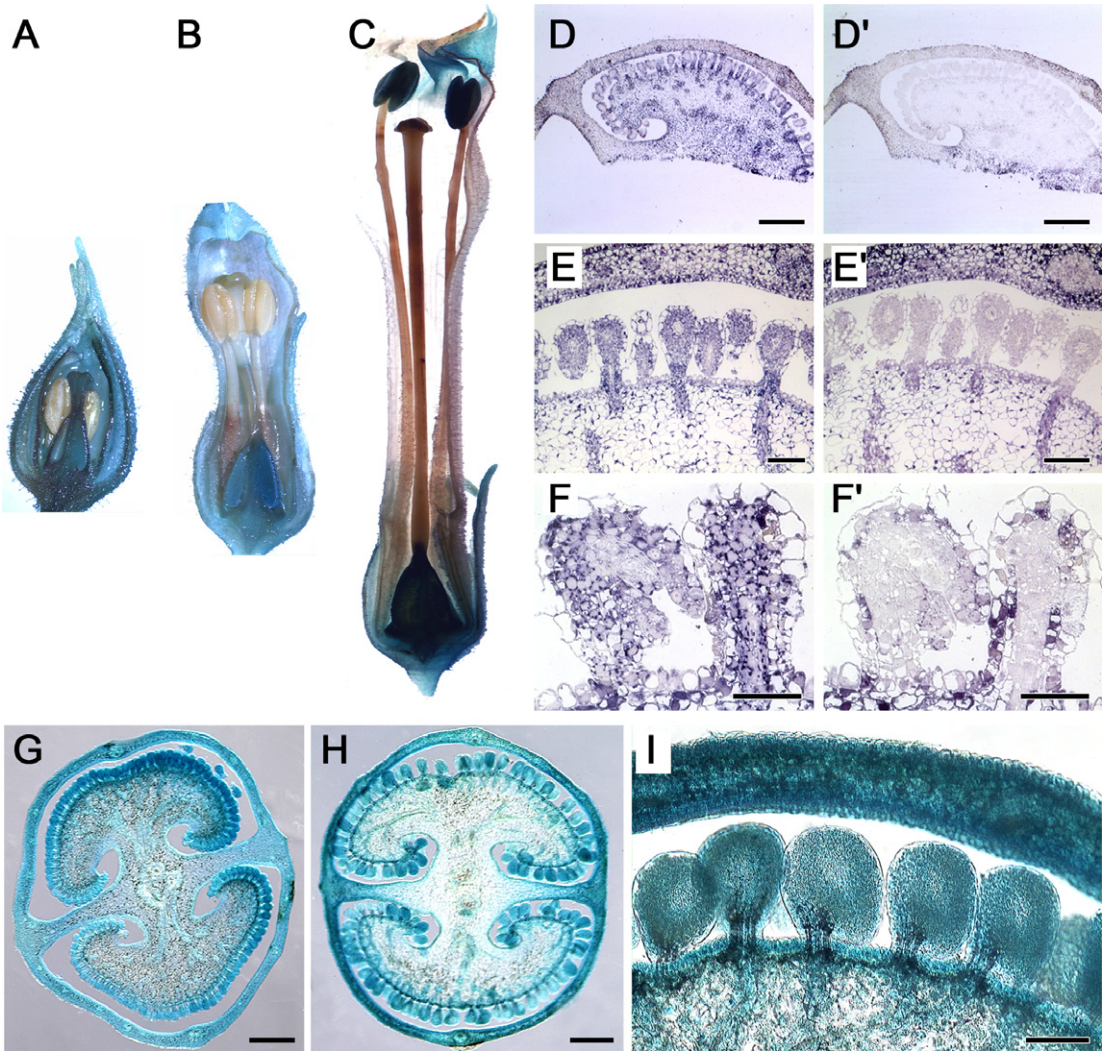

Fig. 5. AOC promoter activity in flowers of tobacco plants carrying a tomato AOC (3600 bp)::uidA construct. (A) Flower bud of 12 mm; (B) flower bud of 25 mm; (C) open flower; (D)–(F) immunolocalization with an anti AOC antibody (tomato) in cross-sections of ovules at different magnification; (D′)–(F′) preimmune serum, (bars in D, D′: 500 μm; in E, E′: 100 μm; in F, F′: 50 μm); (G) cross-section of an ovary of a 12 mm flower bud (bar: 500 μm); (H) cross-section of an ovary of an open flower (bar: 500 μm); (I) higher magnification of cross-section of an open flower (bar: 100 μm).

AOC and late genes such as *PIN2* (Ryan, 2000) based on expression kinetics. The time-dependent shift of AOC(1000 bp) promoter activity from vascular bundles into mesophyll cells suggests that an early gene might be active also at later times including loss of cell-type specific expression. How the *PIN* expression is switched on in mesophyll cells, whereas inducing signals such as JA and systemin are preferentially generated in vascular bundles, is still a matter of discussion (Schilmiller and Howe, 2005). H_2O_2, another inducer of *PIN* expression was suggested to function as a signal between both cell types due to its mobility (Orozco-Cárdenas et al., 2001; Howe, 2004). The AOC promoter activity in mesophyll cells, shown here, suggests JA formation at later times upon wounding, which might be an additional explanation for *PIN2* expression in mesophyll cells.

The AOC(1000 bp)-promoter activity observed upon treatment with JA, systemin or glucose corresponds to that of the activity of the full length 3600 bp promoter as well as to previous expression studies (Stenzel et al., 2003). Furthermore, initial promoter activity originates from vascular bundles (Fig. 3F–I). The comparison of the two different promoter fragments demonstrates that the *cis*-acting elements responsible for JA- and glucose-mediated regulation might be located within the first 1000 bp. This is in agreement with the finding, that conserved *cis*-acting elements assumed to be involved in JA/ET/SA-dependent responses (Adie et al., 2007) are present within 650 bp of 5′-sequences. The untreated fully developed leaflet did not show promoter activity (Fig. 3B and J), but carries AOC protein in vascular bundles (Hause et al., 2000). Presumable, AOC protein is kept here from earlier time in leaf development, when the AOC promoter is active (Fig. 3A).

Local wounding leads to a time-dependent spreading of promoter activity originating from vascular bundles and finally reaching the systemic leaf (Fig. 4A–C). Upon different times following local injection of ^{14}C-labelled JA the main portion was still ^{14}C-JA (Bücking et al., 2004) suggesting that JA is able to move in a similar fashion (Fig. 4D–F). A phloem-specific transport of ^{11}C-JAME in both phloem and xylem pathways was recently documented for tobacco including a common movement of ^{11}C-JAME and photoassimilate in sieve elements (Thorpe et al., 2007). The positron autoradiographs showing distribution of ^{11}C-JAME in tobacco leaves (Thorpe et al., 2007) are very similar to that shown here for ^{14}C-JA in tomato leaves (Fig. 4D–F). These data on transport of JA/JAME and the systemic activation of the AOC promoter as well as the systemic expression of other JA biosynthesis genes such as *LOXD* (Heitz et al., 1997), *AOS* (Howe et al., 2000) or *OPR3*

(Strassner et al., 2002) suggest a systemic activation of JA biosynthesis. But this is not essential for the systemic wound response. Grafting experiments with tomato mutants affected in JA biosynthesis and signaling provided clear evidence, that only JA signaling is required in the systemic leaf (Schilmiller and Howe, 2005; Howe and Jander, 2007; and references therein). This may indicate an invalid regulatory loop in the systemic leaf upon wounding.

3.2. AOC promoter activity during development

In 3-d-old seedlings, AOC promoter activity and AOC proteins were preferentially detected in meristematic cells of the primary root tip (Fig. 1D, I and J). This is consistent with the increased mitotic activity and formation of meristematic cell clusters upon treatment with low (0.1–1 μM) JA concentration (Capitani et al., 2005). Furthermore, AOC promoter activity in the root tip corresponds to elevated levels of JA and JA metabolites (Table 1) and correlates spatially and temporally with the expression of the JA-responsive *AQUAPORIN AthH2* (Kaldenhoff et al., 1995). A similar up-regulation of JA-responsive genes was found by cell-type-specific array analyses in Arabidopsis lines (Birnbaum et al., 2003). In the primary root of 10-d-old seedlings, however, AOC promoter activity appeared preferentially in the elongation zone, which is known to be regulated by auxin. Interestingly, AOC promoter activity was blocked by 10^{-4} M indolyl acetic acid (IAA), which was compromised by the anti-auxin PCIB (Fig. 1O and P). This indicates the repeatedly observed antagonism of auxins and JA. Based on gene expression data for *Arabidopsis thaliana* roots (www.arexdb.org) (Devoto et al., 2005), the following scenario may occur: The coordinate expression of *AOS*, *AOC3* and *OPR3* would increase OPDA and JA levels, which are known to up-regulate the transcription factor *MYC2* in a *COI1*-dependent manner (Lorenzo and Solano, 2005). This cannot occur, if auxin negatively regulates JA biosynthesis, e.g. by down-regulating AOC promoter activity as shown here for tomato. But a lack of MYC2 would compromise its negative role on auxin-inducible *AXR2*/*IAA7* expression, which cannot act anymore as repressors of auxin signaling (Nagpal et al., 2000, 2005). Consequently, the output of IAA-induced decrease of AOC promoter activity could be an amplification in auxin signaling. The increased promoter activity of *CYP97B2* and *CYP79B3*, both of them encode enzymes in auxin biosynthesis, as well as the elevated auxin levels in root tissue of high AOC promoter activity (Ljung et al., 2005) support this scenario. Similar signaling may also occur in tomato. There is at least the homolog of MYC2 in tomato with identical properties (Boter et al., 2004).

The strong AOC promoter activity in ovules of flower buds corresponds to the previously described preferential accumulation of AOC protein and jasmonates in ovaries of tomato (Hause et al., 2000; Miersch et al., 2004). Ovules are known to carry high promoter activity and expression level, respectively, of JA-responsive defense genes such as *PIN2* (Peña-Cortés et al., 1991), *LEUCINE AMINO PEPTIDASE* (*LAP*) (Chao et al., 1999) and *THREONINE DEAMINASE* (*TD*) (Samach et al., 1995). Thus, the glucose-induced AOC promoter activity (Fig. 3I) and preferential AOC promoter activity in ovules (Fig. 2B) support the previously suggested sequence of processes: accumulation of glucose in a sink tissue such as ovules → *AOC* expression and JA formation → expression of JA-inducible defense genes (Hause et al., 2000). The preferential role of JA in ovules is also suggested by the *jai1* phenotype. This JA-insensitive tomato mutant is female sterile, blocked in embryo development and affected in the *JAI1*, the homolog of *COI1* of *A. thaliana* (Li et al., 2004). Additionally, tomato MYC2 has been identified to function as a transcription factor in *PIN2* and *LAP* expression of flowers (Boter et al., 2004) and recent large-scale array analysis of the tomato ovary transcriptome is in accordance with this scenario. The biological relevance of the flower-specific JA-dependent activation of defense genes in tomato has been documented (Damle et al., 2005). The trypsin inhibitory activity of PINs was three orders of magnitude higher in flowers than in leaves, which correlated with a much lower infestation by larvae of *Helicoverpa armigera* in flowers than in leaves.

The similar AOC promoter activities found for developing flowers of transgenic tobacco lines carrying the full length (3600 bp) tomato AOC promoter GUS construct demonstrate that tomato AOC promoter is active even in a heterologous background such as tobacco, and that the 1000 bp fragment tested in the tomato carries already all AOC promoter activities occurring in flower development.

Tomato fruit development is a complex program including generation of carotenoids and volatiles. Several of them are known to be JA-inducible. In this respect, it is interesting to note that seeds of ripe fruits contain a 4-fold higher amount of octadecanoids and jasmonates than the pericarp, and that 12-OH-JA and OPDA are the dominant compounds (Wasternack et al., 2007). Since 12-OH-JA is formed in a JA-dependent manner (Miersch et al., 2008), the JA-forming capacity has to be high. The preferential AOC promoter activity in seeds of developing fruits (Fig. 2K) accords with such assumption.

The tissue- and organ-specific AOC promoter activity data shown here support already published data on JA levels and JA responsive gene expression. Back crosses of the AOC promoter GUS lines into a JA-deficient (*spr2*) – and JA-insensitive (*jai1*) – mutant background are underway to further analyze the role of spatial and temporal pattern of AOC promoter activities in tomato development and responses to environmental cues.

4. Experimental

4.1. Isolation of the AOC promoter and construction of the cassettes for transformation

To isolate the 5′-sequences responsible for regulating expression of the AOC, genome walks were performed using the *Genome Walker Kit* (Clontech) according to the manufacturer's instruction on genomic libraries of tomato starting from exon I of AOC. A 3600 bp fragment comprising the complete AOC promoter was obtained by a single genome walk (NCBI GenBank accession Number EU 437573).

The HindIII/EcoRI fragment of the vector pBI121 carrying the *GUS* gene, the 35S promoter and the *NOS* terminator was cloned into the vector pGreen 0029 (Roger et al., 2000) (http://www.pgreen.ac.uk/). Unwanted restriction sites within the polylinker of p35S-GUS-pGreen 0029 were cut with EcoRI, refilled at the overlaying ends with Klenow polymerase and religated. This vector p35S-GUS-pGreen 0029-2 was used to construct the transformation cassettes. For that 35S-GUS-pGreen0029-2 was digested with BamHI, refilled at the overlaying ends with Klenow polymerase and than digested with SalI. The initially isolated full length tomato AOC promoter (3600 bp) was cut with SalI and MlsI. The resulting plasmid was named *SlAOC-3600 bp::GUS*. In case of the *SlAOC-1000bp::GUS* construct, the clone *SlAOC-3600::GUS* was restricted with XhoI and HindIII, refilled with Klenow polymerase and religated.

4.2. Plant material and generation of transgenic lines

Transgenic *Solanum lycopersicum* cv. MicroTom and cv. Moneymaker plants and transgenic *N. tabacum* cv. *Samsun* NN plants were generated by *Agrobacterium*-mediated gene transfer, using *Agrobacterium tumefaciens* strain LBA4404 (tobacco) and GV3101 (MicroTom), essentially according to Zambryski et al. (1983), Filiat-

ti et al. (1987), and (Horsch et al., 1985), respectively. The independent transformants were screened on 1 × Murashige and Skoog medium including vitamins containing 30 μg/ml kanamycin, 0.16 μg/ml betabactyl, 3% sucrose, 0.1 μg/ml IAA and 0.8% agar. Kanamycin resistant plants were transferred to soil and grown in the greenhouse together with the corresponding wild types (Wasternack et al., 1998).

For Southern analysis, seeds of F1 plants were surface sterilized for 10 min in 70% ethanol, for 10 min in 6% NaOCl and 0.1% Triton X-100, washed 5 times in sterile distilled water and were plated on 1 × Murashige and Skoog medium including vitamins containing 3% sucrose, 30 μg/ml kanamycin, 0.16 μg/ml betabactyl and 0.8% agar (Duchefa, Haarlem, NL). From 30 kanamycin-resistant primary tomato (Moneymaker) transformants that proved to harbour the 1000 bp and 3600 bp, respectively, AOC promoter-GUS fusion by PCR analyses, three independent lines were selected for further analyses. In case of primary tobacco transformants, four lines carrying the full length tomato AOC promoter each of them as GUS fusion were generated as independent lines and proved by PCR analyses. After initial tests on GUS activity of the primary transformants, one and three independent transgenic tomato lines carrying the 1000 bp fragment and the full length (3600 bp), respectively, tomato AOC promoter in front of the GUS gene, and four independent transgenic tobacco lines carrying the full length tomato AOC promoter in front of the GUS gene were subjected for further analyses.

4.3. Histochemical analysis of GUS activity

Histochemical staining of plant tissue for GUS activity was performed according to the method of Jefferson et al. (1987). Tissue samples were placed in substrate solution (100 mM sodium phosphate, pH 7.0, 2 mM 5-bromo-4-chloro-3-indolyl-glucuronide, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA and 0.1% [v/v] Triton-X100), vacuum-infiltrated for 5 min, and then incubated at 37 °C for 18 h. Subsequently, the samples were transferred to 70% ethanol to remove chlorophyll. For sections stained plant organs (leaves, root tips and flower buds) were embedded in paraplast (Sigma, Teichenhofen, Germany), sectioned, deparaffinized and rehydrated as described (Hause et al., 2003).

4.4. Treatments and wounding experiments

Seeds of T2 plants carrying 2–3 copies of the 1000 bp AOC promoter GUS construct and T1 plants carrying 2–3 copies of the 3600 bp promoter GUS construct were transferred to soil and grown in the greenhouse. Three week-old plants were tested on GUS activity upon wounding of a detached leaflet with a forceps, floating on distilled water for 2 h and an overnight-GUS staining. Selected plants showing similar GUS activity were used 8 days later for application experiments as well as analysis of the local and systemic response.

Treatments of leaves were performed with 50 μM JA, 100 pM systemin, 0.5 M glucose or 100 mM phosphate buffer as the control by feeding *via* the freshly cut petiole of a detached leaf. Wounding was performed by crushing each leaflet of a tomato leaf once or a tobacco leaf three times across the mid vein with a tweezer. Treatment of primary roots of 7-d-old plants were performed with 100 mM phosphate buffer (control), 10 μM JAME, 100 μM IAA or 100 μM PCIB for 24 h before GUS-staining.

In wound experiments, detached leaves of 4-weeks-old plants grown in a greenhouse were wounded with a forceps, floated on distilled water for 2 h and 18 h, respectively, and were analyzed by overnight-GUS staining. To compare the local and systemic response, a lower fully developed leaf of a 4-weeks-old plant grown under sterile conditions on agar was wounded with a forceps 1–2 times across the mid vein. After 2 h and 8 h, respectively, whole plants were analyzed by overnight-GUS staining.

4.5. Feeding of [^{14}C]-JA and whole plant autoradiography

For whole plant autoradiography, 20 μl of a solution containing 5 mM [^{14}C]-JA (about 3500 bq) were pressure-infiltrated into the upper leaflet of a lower leaf of 4-week-old plants. Infiltrated leaves were subsequently wounded across the midvein with a hemostat at the infiltrated area. At indicated times after application of [^{14}C]-JA, shoots of plants were excised at the base of the stem with a razor blade and lightly pressed onto filter paper. The shoots were placed on a slab-gel dryer, vacuum dried for 3 h at 50 °C according to Narváez-Vásquez et al. (1995) and then positioned directly against a low energy storage phosphor screen (Molecular Dynamics, Ismaning, Germany). Images with a resolution of 50 μm were obtained using Storm Phosphoimager (Molecular Dynamics) equipped with the ImageQuant software. Non-radioactive labeled plants used as controls did not show any signal.

4.6. Immunocytochemical analysis of AOC location

Tissues of tomato and tobacco plants were fixed with 4% paraformaldehyde/0.1% (v/v) Triton X-100 in phosphate buffered saline (PBS) immediately after harvest and embedded in polyethylene glycol as described previously (Hause et al., 1996). Immunodecoration and visualization of AOC was performed with an antibody raised against recombinant tomato AOC according to Hause et al. (2000).

4.7. Analysis of JA, OPDA and JA-metabolites

Root tips 1 cm in length and roots of 7-d-old seedlings were harvested by rapid cutting up to an amount of 200 mg fresh weight and immediately frozen in liquid nitrogen. Extraction, purification and quantification of JA, OPDA and the JA-metabolites 12-OH-JA, 12-*O*-Glc-JA and 12-HSO_4-JA were performed as described (Miersch et al., 2008).

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