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Occurrence of the allene oxide cyclase in different organs and tissues of *Arabidopsis thaliana*

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Abstract

Occurrence of an essential enzyme in jasmonate (JA) biosynthesis, the allene oxide cyclase, (AOC) was analyzed in different developmental stages and various organs of *Arabidopsis thaliana* plants by immuno blot analysis and immunocytological approaches. Levels of AOC and of the two preceding enzymes in JA biosynthesis increased during seedling development accompanied by increased levels of JA and 12-oxophytodienoic acid levels after 4 and 8 weeks. Most tissues including all vascular bundles and that of flower buds contain AOC protein. Flowers shortly before opening, however, contain AOC protein preferentially in ovules, stigma cells and vascular bundles, whereas in anthers and pollen AOC could not be detected. The putative roles of AOC and JA in development are discussed.

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Keywords: Jasmonates; Allene oxide cyclase; JA biosynthesis; Development; Immunocytochemical localization; *Arabidopsis*

1. Introduction

Different lipid-derived signals are formed when plants adjust to biotic and abiotic stresses. Jasmonates are most prominent among these signals. The term “jasmonates” is commonly used for jasmonic acid (JA), its methyl ester (JAME) and derivatives such as JA amino acid conjugates, whereas the precursor 12-oxo-phytodienoic acid (OPDA) and its derivatives are designated as “octadecanoids”. All oxidation products of polyunsaturated fatty acids (PUFAs) are designated as “oxylipins”.

Jasmonates are synthesized by one of seven different branches of the lipoxygenase (LOX) pathway which is initiated by the LOX-catalyzed oxygenation of α-linolenic acid (α-LeA) (Feussner and Wasternack, 2002). Insertion of oxygen can take place 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 (13*S*-hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic acid, 13-HPOT) (Fig. 1). 13-HPOT can be metabolized by an allene oxide synthase (AOS) into an unstable allene oxide which is cyclized by an allene oxide cyclase (AOC) to *cis*(+)-OPDA (9*S*,13*S*) carrying the enantiomeric structure of the naturally occurring JA. Subsequent reduction of the cyclopentenone ring and three steps of β-oxidation lead to (+)-7-*iso*-JA which equilibrates to the more stable (-)-JA (Fig. 1).

Except for β-oxidation, all genes encoding enzymes of JA biosynthesis have been cloned from several plants including *Arabidopsis* (Creelman and Rao, 2002; Feussner and Wasternack, 2002). Among the six cDNAs coding for LOXs of *A. thaliana* (Bell and Mullet, 1993; Melan et al., 1993; Feussner and Wasternack, 2002), several might be involved in JA biosynthesis. AOS is encoded by a single copy gene (Laudert et al., 1996), whereas for AOC, four different genes were identified (Stenzel et al., 2003b). Among the three OPDA reductases (OPRs) of *A. thaliana*, only the OPR3 is converting specifically *cis*-(+)-OPDA (Müssig et al., 2000; Sanders et al., 2000; Schaller et al., 2000).

Abbreviations: α-LeA, α-linolenic acid, AOC, allene oxide cyclase, AOS, allene oxide synthase, JA, jasmonic acid, LOX, lipoxygenase, OPDA, 12-oxophytodienoic acid, OPR, OPDA reductase, PUFA, polyunsaturated fatty acid

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13-LOXs, AOS and AOCs of *A. thaliana* carry a putative target sequence for chloroplast import, and chloroplast location for all three enzymes were shown immunocytochemically and by import studies, respectively (Bell et al., 1995; Laudert et al., 1996; Stenzel et al., 2003b). OPR3 carries a peroxisomal target sequence (Stintzi and Browse, 2000) and was detected immunocytochemically in peroxisomes (Strassner et al., 2002).

Transcripts coding for LOXs, AOS, AOCs and OPRs of *A. thaliana* accumulate upon wounding, pathogenic attack or other stresses (Reymond et al., 2000; Strassner et al., 2002; Stenzel et al., 2003b), and most plants analyzed so far accumulate JA transiently within the first hour upon wounding or other external stimuli (Stintzi et al., 2001; Stenzel et al., 2003b). The JA biosynthesis seems to be regulated by substrate availability, since leaves of transgenic plants overexpressing *AOS* or *AOC* did not show constitutively elevated JA levels, but form more JA than the wild type upon wounding (Wang et al. 1999; Laudert et al., 2000; Stenzel et al., 2003a). Since expression of all corresponding genes are JA-inducible, a feed forward regulation of JA biosynthesis is also supposed. As shown by comparison to the wild type, the JA-deficient mutant *opr3* contains less AOC but unchanged level of AOS and LOX, respectively. Therefore, the AOC seems to be the preferential target in regulation of JA biosynthetic capacity (Stenzel et al., 2003b).

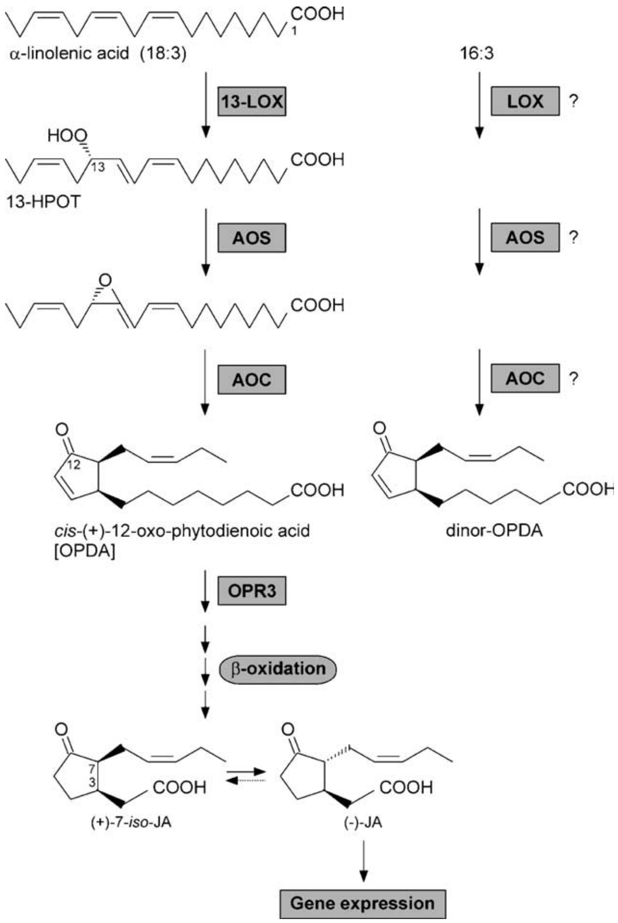

Fig. 1. Scheme of JA biosynthesis.

The formation of JA and OPDA and their role in plant responses to biotic and abiotic stresses is well-documented (reviewed in Wasternack and Hause, 2002). Less is known about biosynthesis of JA during development (Creelman and Mullet, 1997). Several mutants of *A. thaliana* affected in JA biosynthesis indicate a role of JA in flower development: (i) The mutant *dad1*, defective in a chloroplast located phospholipase A1, is JA-deficient and male sterile (Ishiguro et al., 2001). (ii) The mutants *opr3* and *dd1*, both of them defective in *OPR3*, are male sterile and affected in proper pollen development (Sanders et al., 2000; Stintzi and Browse, 2000). (iii) Expression of *AOS* (Kubigsteltig et al., 1999) and *OPR3* (Sanders et al., 2000) in distinct stages of flower development suggest an altered JA biosynthetic capacity during flower formation. Furthermore, tissue-specific *AOS* expression and stage-specific alteration of JA levels has been observed for soybean and barley (Creelman and Mullet, 1995; Maucher et al., 2000).

Recently, LOX, AOS and AOC protein was shown to occur abundantly in fully developed rosette leaves of *A. thaliana* but much less in young developing leaves (Stenzel et al., 2003b). Here, we show that during development the JA biosynthetic capacity increased in terms of LOX, AOS and AOC protein content and altered levels of JA/OPDA/dinor-OPDA. Immunocytological analysis revealed an occurrence of AOC in all flower tissues except anthers and pollen.

2. Results and discussion

2.1. LOX, AOS and AOC protein accumulate steadily during development

The occurrence of LOX, AOS and AOC protein was analyzed by immunoblot analysis in different stages of seedling development and of rosette leaves. Already one week after imbibition, all three enzymes were detectable (Fig. 2A). LOX protein was maximal in 4-weeks-old seedlings, whereas the AOS protein increased steadily even in leaves of 9–14-weeks-old plants. A steady increase appeared also in the amount of AOC protein. The corresponding mRNAs for AOS and AOC were detectable after three weeks and decreased after 11 weeks (Fig. 2B). Among the six LOX sequences identified in *A. thaliana* (Melan et al., 1993; Bell et al., 1995; Feussner and Wasternack, 2002), at least LOX2 (Bell et al., 1995), LOX3 and LOX4 (Feussner, unpublished) are candidates to function in JA biosynthesis. With the polyclonal anti-LOX-antibodies used here only the bulk of LOX proteins was detected. Due to the preferential occurrence of LOX2 (Bell et al., 1995), the immuno blot analysis shown here is at least partially indicative for JA-related LOX protein during development. In case of AOS, a single copy gene product, the anti-AOS-antibodies should reflect the actual AOS protein level. For AOC, most presumably the detected protein shown in Fig. 2A is preferentially indicative for AOC2. Among all four recombinant AOCs, AOC2 was preferentially recognized by the anti-AOC antibodies, and the *AOC2* gene is most actively expressed (Stenzel et al., 2003b).

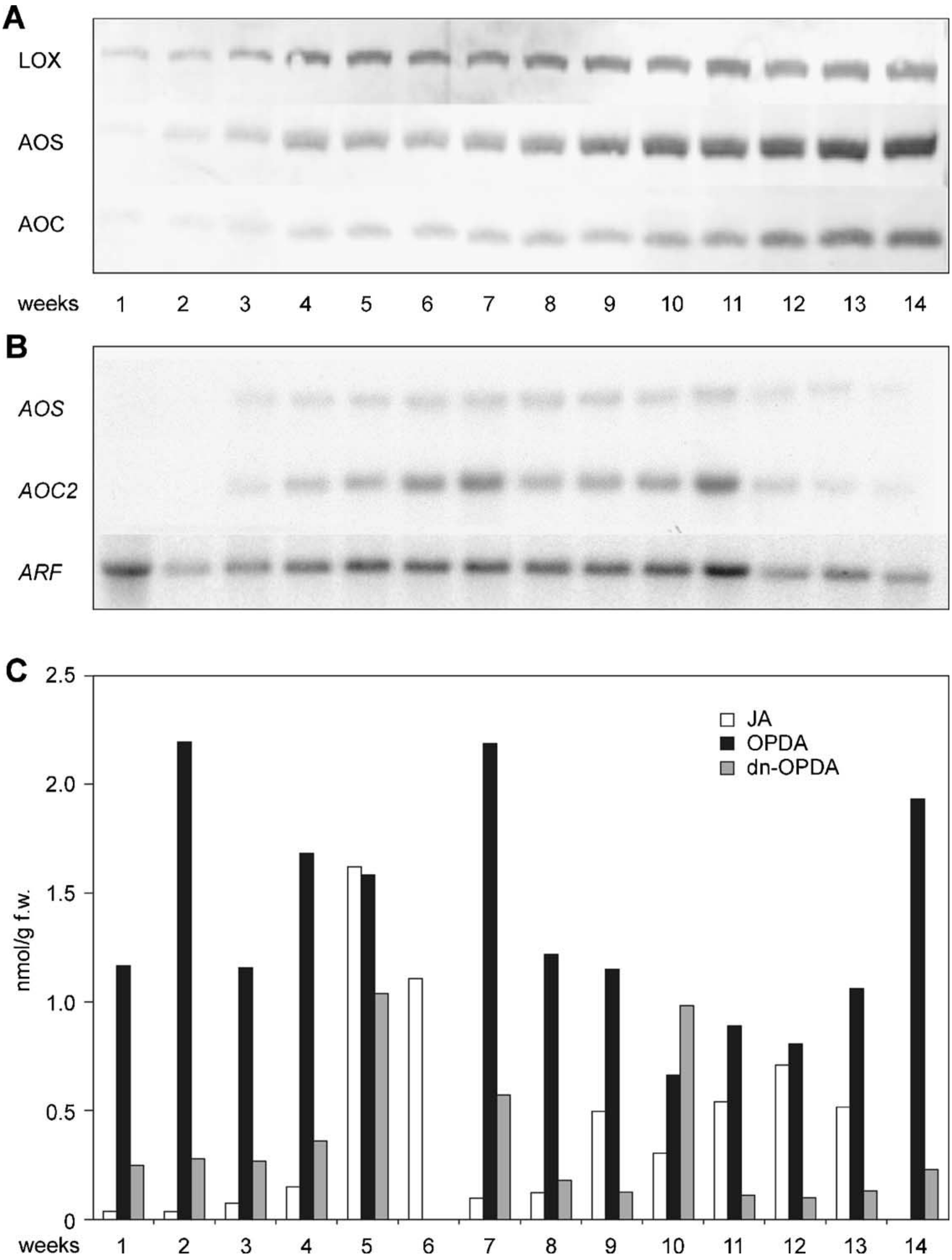

Fig. 2. Immunoblot analysis (A), Northern blot analysis (B) and content of JA, OPDA and dinor-OPDA (C) during development of *A. thaliana* ecotype Columbia. Aliquots of identical leaf material pooled from 70 to 100 plants were subjected to extraction of proteins, total RNA and the oxylipins. Per lane 20 µg total RNA and 5 µg total protein, respectively, were loaded. RNA loading was checked by probing with an ARF probe. Blots were hybridized with a ^{32}P-labeled full-length cDNA of AOC2 and AOS, respectively. Immuno blots were probed with anti-LOX antibodies at a dilution of 1:1000, with anti-AOS antibodies at a dilution of 1:2500, and with anti-AOC2 antibodies at a dilution of 1:5000.

These data indicate a steadily increased capacity for JA biosynthesis during development of *A. thaliana* plants. Quantitative measurements of OPDA, dinor-OPDA and JA revealed a constitutive high level of OPDA. JA levels peaked in 5- to 6-weeks-old plants and increased again in plants older than 8 weeks (Fig. 2C). Dinor-OPDA exhibited an average level of about 200–300 pmol per g f.w., but reached also levels of 1 nmol per g f.w. in 5- and 10-weeks old plants. These data are in accordance with the previously described positive feed back loop in JA biosynthesis (Sasaki et al., 2001; Stenzel et al., 2003b). Whereas the wild type is able to accumulate increasing amounts of JA biosynthetic enzymes and JA during development (Figs. 2A and C), the JA-deficient mutant *opr3* accumulates much less AOC protein in fully developed leaves (Stenzel et al., 2003b). The remarkable high level of free OPDA throughout the development of *A. thaliana* corresponds to previously described levels of 1–2 nmol per g f.w. in untreated fully developed leaves (Laudert and Weiler, 1998; Müller et al., 2002; Stintzi et al., 2001). Beside this amount of free OPDA, *A. thaliana* leaves carry an up to 10-fold higher amount of OPDA esterified to the α-LeA moiety of lipids (Stelmach et al., 2001). This esterified OPDA unknown in function to date is, however, released upon wounding.

LOX-, AOS- and AOC-encoding genes of *A. thaliana* are inducible by OPDA as analyzed by treatment with

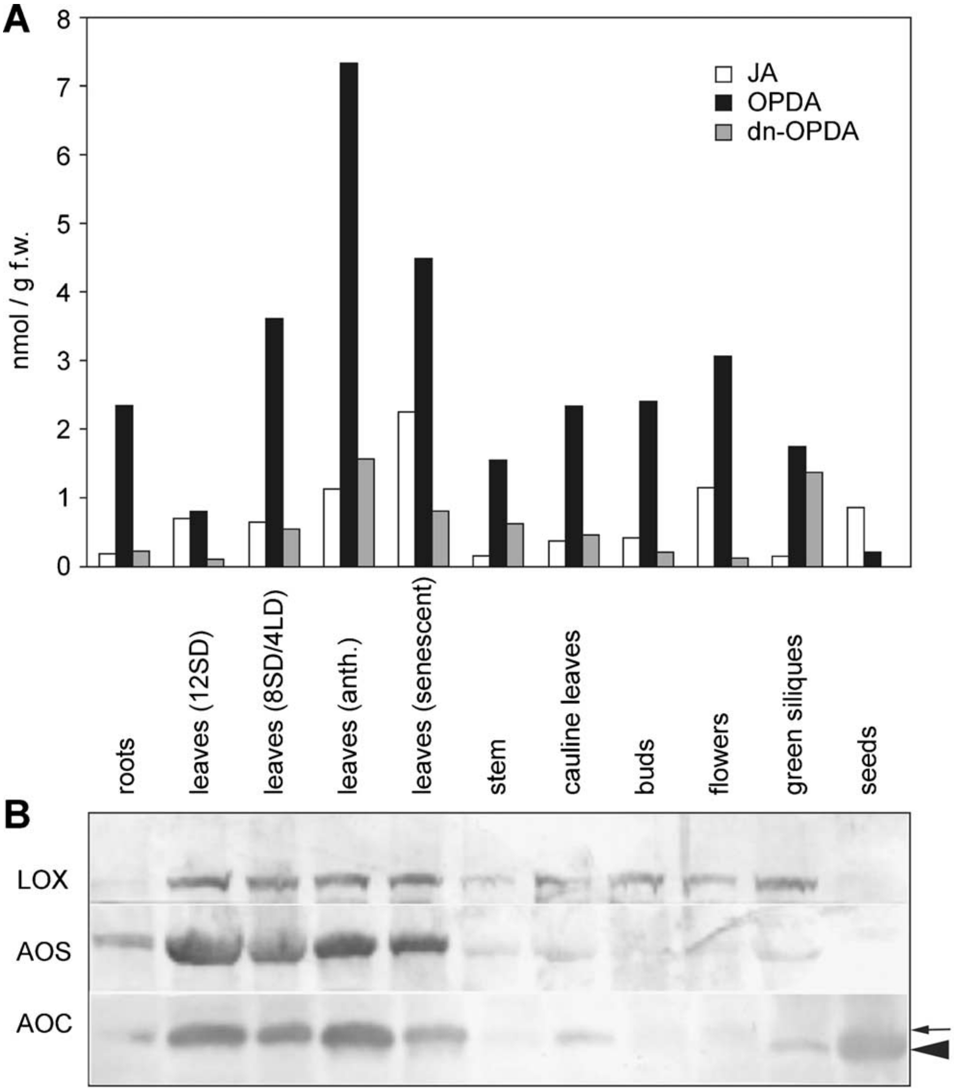

Fig. 3. Content of JA, OPDA and dinor-OPDA (A) and immunoblot analysis (B) in different organs and developmental stages of *A. thaliana* ecotype Columbia. Aliquots of identical tissues pooled from 70 to 100 plants were subjected to extraction of oxylipins and proteins. Per lane 5 μg total protein were loaded, and blots were probed as described in the legend of Fig. 2. In protein extracts of dry seeds and less in that of green siliques, AOC protein could not be detected properly, since the anti-AOC-antibodies cross-react with protein(s) lower in kDa than the AOC. This cross-reactivity appeared already with the pre-immune serum.

OPDA (Laudert and Weiler, 1998; Stintzi et al., 2001; Stenzel et al., 2003b). The endogenous OPDA, however, might be unable to express genes due to its confinement to the plastids, but is coming active in gene expression upon wounding (Stintzi et al., 2001).

2.2. LOX, AOS and AOC accumulate preferentially in leaves

Immunoblot analysis of total proteins, extracted from various organs revealed abundant appearance of LOX, AOS and AOC in rosette leaves of different age (Fig. 3B). The high levels are kept under long day conditions. Also anthocyan-accumulating rosette leaves and senescent leaves carry high level of LOX, AOS and AOC protein. Corresponding high levels were found in these leaves for OPDA and JA (Fig. 3A). These OPDA and JA levels are in accordance with data detected by the recently described multiplex GC-MS/MS technique for acidic phytohormones (Müller et al., 2002), where similar levels were found in fully developed leaves, stems and cauline leaves. The role of JA in senescence was suggested for *A. thaliana* leaves by transcriptional upregulation of genes encoding LOX, AOS, AOC and OPR as well as by elevated JA levels in senescent leaves (He et al., 2002). The high JA levels shown here for fully senescent leaves is in line with that. The accumulation of other oxylipins is also senescence-associated in *A. thaliana* (Berger et al., 2001). These oxylipins, however, were formed autoxidatively, as indicated by a decline in their *S*/*R* ratio between early and late stages of leaf development. A link between anthocyanin synthesis and JA, first described for soybean (Staswick et al., 1992), is indicated by the high endogenous levels of JA and OPDA in anthocyanin-containing rosette leaves (Fig. 3A). In long term processes such as leaf development the amount of LOX, AOS and AOC protein may allow the plants overtime to accumulate jasmonates leading to accumulation of metabolites formed JA-dependently such as anthocyanins.

Roots, stems, cauline leaves, flower buds, flowers and green siliques exhibited low or undetectable amounts of LOX, AOS and AOC protein in the immuno blot analysis (Fig. 3B). The immunocytological inspection, however, for AOC (see below) clearly indicates occurrence of AOC in all vascular bundles, thus being in accordance with the levels of OPDA and JA in these organs (Fig. 3A). In case of roots, the JA-biosynthetic capacity is also indicated by a raise in JA observed upon infection with the root-pathogen *Fusarium oxysporum* ssp. *matthiolae* (Bohlmann et al., 1998). Dry seeds contrast from other organs by a residual amount of OPDA and higher amount of JA, which may attribute to the effect of JA on seed germination (Koda, 1997), and together with other factors such as abscisic acid, JA may sustain seed germination inhibition.

2.3. AOC protein accumulates in all vascular bundles

The increase in AOC protein levels during *A. thaliana* development prompted us to inspect different vegetative organs immunocytochemically (Fig. 4). In fully developed rosette leaves the vascular bundles and the surrounding mesophyll cells contained AOC protein (Figs. 4A and B). Due to the location of AOC within chloroplasts (Stenzel et al., 2003b), the label indicative for AOC is not randomly distributed but highly localized within the cells. A residual label appeared with the preimmune serum (Fig. 4A). In stem tissues (Figs. 4C and D), the phloem and the surrounding green tissues contained AOC, whereas the preimmune serum revealed only some cross-reactivity (Fig. 4C). In primary roots, the central cylinders and the cortex cells accumulated significant amount of AOC (Figs. 4E and F), whereas the preimmune serum gave cross-reactivity only with some epidermal cells of the root (Fig. 4E). At higher magnification label was detectable even in phloem cells and xylem parenchyma cells (Fig. 4F). These data indicate occurrence of AOC protein in most if not all cell types of vegetative tissues.

2.4. Flower buds contain AOC protein randomly, but in flowers shortly before opening AOC appears preferentially in ovules, stigma cells and vascular bundles, whereas anthers and pollen lack AOC

The low or even undetectable amount of AOC protein found by immunoblot analysis of flowers contrasts from the remarkable OPDA and JA levels detected in flowers (Fig. 3A versus B). For a detailed inspection of the occurrence of AOC protein in flowers, we analyzed immunocytochemically longitudinal and transverse sections of flowers at different developmental stages (Figs. 5A–H). The overall view of an inflorescence showed label in vascular bundles and parenchyma cells, as well as in the petals, filaments and anthers of very young flower buds (Fig. 5A). The corresponding longitudinal sections probed with the preimmune serum revealed minute cross-reactivity in anthers of the buds (Fig. 5A). A more detailed inspection of this very early stage of flower development showed label in cells of the ovule primordia including the microgametophyte mother cells and the parietal layer surrounding them (Figs. 5B and C), but negligible cross-reactivity with the preimmune serum (Fig. 5B). In flowers shortly before opening, however, label appeared more specifically. The anthers including the pollen were free of label in longitudinal (Fig. 5D) and cross-sections (Fig. 5F), whereas the filaments both longitudinally and transversely sectioned showed label (Figs. 5D and F). Again, in vascular bundles and the surrounding parenchyma cells of petals and sepals occurrence of AOC was indicated (Figs. 5D and E). A strong label indicating AOC was detected in the ovaries, within the pistil (Figs. 5D, E and G) and in stigma cells as well as the enlarged papillar cells (Figs. 5D and H). All corresponding tests with the preimmune serum revealed residual or no label (Figs. 5D , E and H). Summarizing, the AOC protein occurs in all cell types and tissues of flower primordial. In flowers shortly before opening, however, AOC was undetectable in anthers and pollen and appeared preferentially in ovules, stigma cells and vascular bundles.

These findings correspond only partially with AOS promoter activity data analyzed with *AOS::uidA* plants (Kubigsteltig et al., 1999). Since GUS activity data are not directly comparable with immunocytological data, we inspected for comparison the occurrence of AOS protein in flower tissues immunocytologically. An interesting difference between AOS and AOC became obvious for the petal abscission zone, the anthers and pollen. The AOS appeared abundantly in the petal abscission zone and the mature pollen (Figs. 5I and K), whereas a residual amount was found with the corresponding preimmune serum (Figs. 5I and K). These results correspond to the GUS activity data detected with the *AOS::uidA* plants (Kubigsteltig et al., 1999), but contrasts from the occurrence of AOC. We can not rule out that AOC protein which is clearly detectable in microgametophyte mother cells (Figs. 5B and C) becomes below the detection limit in mature pollen and anthers. The detection of AOC protein in microgametophyte mother cells and AOS promoter activity in anther development (Kubigsteltig et al., 1999) suggest that these tissues have the capacity to form OPDA or even JA. An essential role of JA in anther and pollen

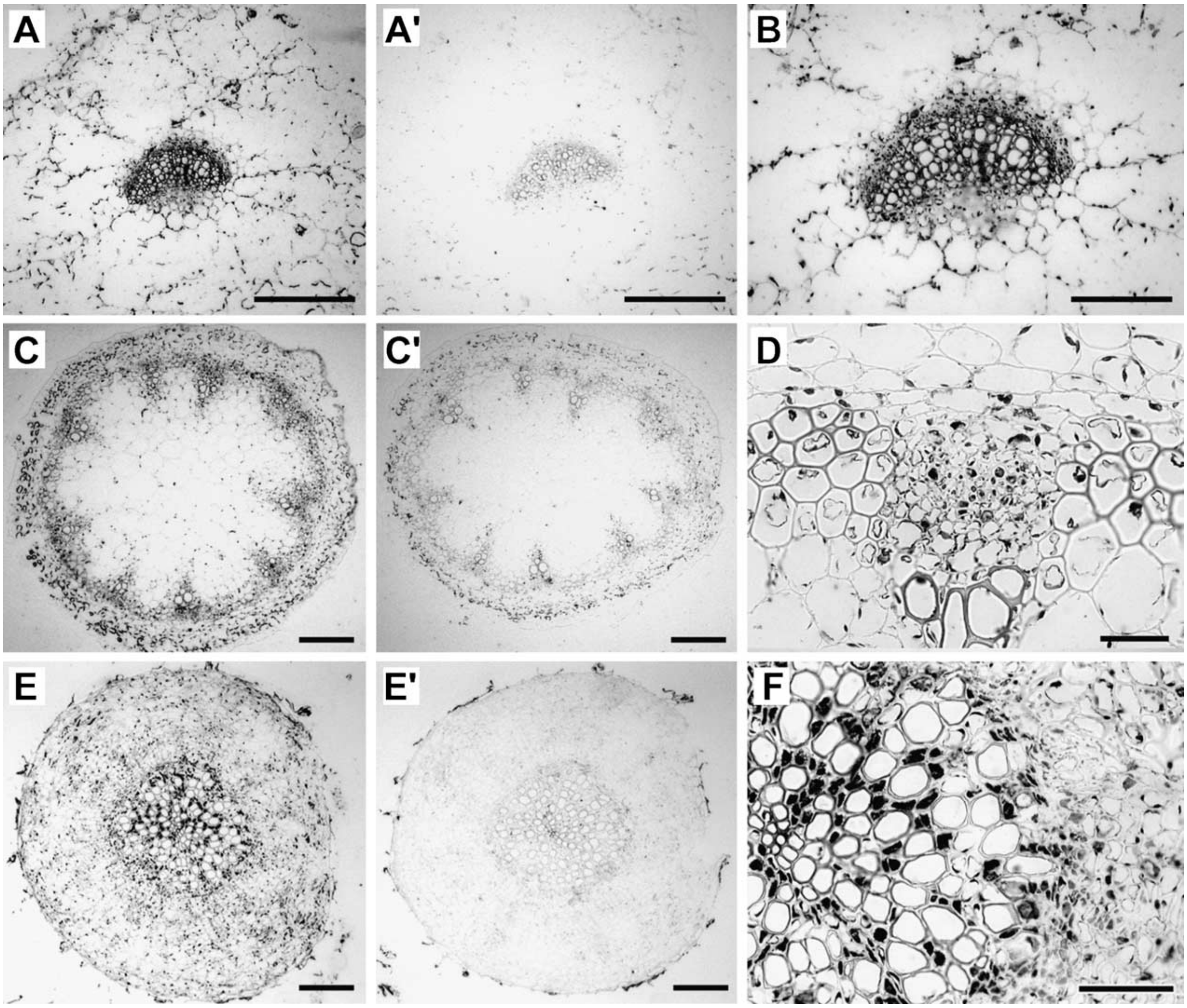

Fig. 4. Distribution of AOC protein in vegetative organs of *A. thaliana*. Roots from 3-weeks-old plants, leaves and stems from 8-weeks-old plants were used for immunolabeling as described in methods. A-F immuno stain, A , C , E controls performed with pre-immune serum. A, A main vein of a rosette leaf, bar = 200 μm. B higher magnification of A showing the vascular bundle, bar = 100 μm. C, C cross section of stem, bar = 100 μm. D higher magnification of C showing a vascular bundle, bar = 25 μm. E, E cross section of the primary root, bar = 100 μm. F higher magnification showing the central cylinder, bar = 50 μm.

development is clearly indicated by several male sterile mutants. The JA-deficient *fad3-2fad7-2fad8* and JA-deficient mutants *opr3* and *dd1* are affected in a proper release of pollen from the tapetum (McConn and Browse, 1996; Sanders et al., 2000; Stintzi and Browse, 2000). Male-sterility occurs also in the JA insensitive mutant *coi1* indicating JA-dependent processes in pollen development (Feys et al., 1994). Furthermore, a mutation of the single copy gene AOS is male sterile (Park et al., 2001).

The JA-deficient mutant *dad1* has an interesting flower-phenotype (Ishiguro et al., 2001). In *dad1* flowers the elongation of stomium filaments is delayed before flower opening. Consequently, *dad1* is male sterile, since the self-pollination, characteristic for *Arabidopsis* flowers cannot occur. These data led to the hypothesis that JA-regulated water uptake into stamen filaments might be responsible for synchronization of pollen maturation, anther dehiscence and flower opening (Ishiguro et al., 2001). The occurrence of AOC protein in stamen filaments (Figs. 5D and F) supports that these tissues may generate JA.

The occurrence of AOC in ovules (Figs. 5E and G) is in accordance with its abundant and specific occurrence in corresponding tissues of tomato (Hause et al., 2000). JA is not a limiting factor for ovule development, since all mutants mentioned above are maternally fertile. JA-inducible genes, however, coding for defense proteins

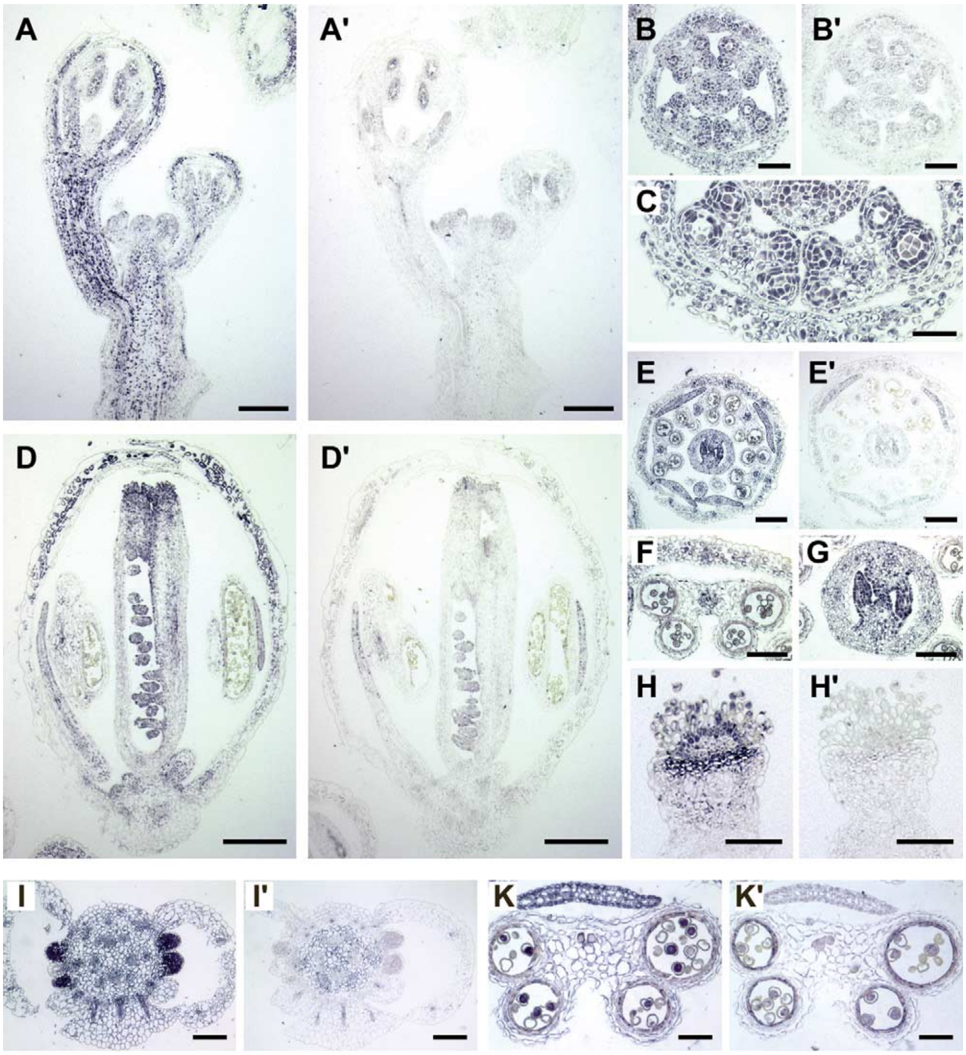

Fig. 5. Distribution of AOC protein (A–H) and of AOS protein (I–K) in the organs and different developmental stages of 8-weeks-old flower buds of *A. thaliana*. A–K immuno stain, A , B , D , E , H , I , K controls performed with pre-immune serum. A, A flower meristem and young flower buds, longitudinal section, bar = 100 μm. B, B young flower bud, cross section, bar = 50 μm. C higher magnification of B, bar = 25 μm. D, D flower bud with developing ovules, longitudinal section, bar = 100 μm. E, E flower bud as in D, cross section, bar = 100 μm. F higher magnification of E showing an anther, bar = 50 μm. G higher magnification of E showing the ovary, bar = 50 μm. H, H stigma of a fully developed flower bud shortly before anthesis, bar = 100 μm. I, I cross section near the bottom of a flower bud with petal bases, bar = 100 μm. K, K cross section of anthers with mature pollen, bar = 25 μm.

such as PINs, γ-thionins, defensins, and enzymes of phytoalexin synthesis were found to be expressed preferentially in ovules and epidermal cell layer of flower organs (Lantin et al., 1999; Lay et al., 2003; Peña-Cortés et al., 1991). It will be interesting to see, whether the antifungal activity detected recently for a flower-specific defensin (Lay et al., 2003) increases the defense status of flowers.

The strong occurrence of AOC protein in stigma cells and the enlarged papillar cells (Figs. 5D and H) points to some specific JA formation in these tissues. Due to remarkable amount of jasmonates in mature pollen (Miersch et al., 1998; Yamane et al., 1982), JA may function in pollen germination, pollen tube growth and/or synthesis of phytoalexins known to be formed in the stigma upon pollination (Vogt et al., 1994).

Among the accumulating data of microarray analyses, there are several examples of JA dependent expression of JA/ethylene-responsive transcription factors such as ERF1 and ERF2 (Lorenzo et al., 2003; Schenk et al., 2000; Spoel et al., 2003). I--T*[Nh;gal-*ha.l-Da-n.m)8Dk