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RESEARCH PAPER

ALLENE OXIDE CYCLASE (*AOC*) gene family members of *Arabidopsis thaliana*: tissue- and organ-specific promoter activities and *in vivo* heteromerization*

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* Dedicated to Professor Benno Parthier, who inaugurated the molecular analysis of mode of action of jasmonates, on the occasion of his 80th birthday.

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Abstract

Jasmonates are important signals in plant stress responses and plant development. An essential step in the biosynthesis of jasmonic acid (JA) is catalysed by ALLENE OXIDE CYCLASE (AOC) which establishes the naturally occurring enantiomeric structure of jasmonates. In *Arabidopsis thaliana*, four genes encode four functional AOC polypeptides (AOC1, AOC2, AOC3, and AOC4) raising the question of functional redundancy or diversification. Analysis of transcript accumulation revealed an organ-specific expression pattern, whereas detailed inspection of transgenic lines expressing the GUS reporter gene under the control of individual *AOC* promoters showed partially redundant promoter activities during development: (i) In fully developed leaves, promoter activities of *AOC1*, *AOC2*, and *AOC3* appeared throughout all leaf tissue, but *AOC4* promoter activity was vascular bundle-specific; (ii) only *AOC3* and *AOC4* showed promoter activities in roots; and (iii) partially specific promoter activities were found for *AOC1* and *AOC4* in flower development. *In situ* hybridization of flower stalks confirmed the GUS activity data. Characterization of single and double *AOC* loss-of-function mutants further corroborates the hypothesis of functional redundancies among individual AOCs due to a lack of phenotypes indicative of JA deficiency (e.g. male sterility). To elucidate whether redundant *AOC* expression might contribute to regulation on AOC activity level, protein interaction studies using bimolecular fluorescence complementation (BiFC) were performed and showed that all AOCs can interact among each other. The data suggest a putative regulatory mechanism of temporal and spatial fine-tuning in JA formation by differential expression and via possible heteromerization of the four AOCs.

Key words: *ALLENE OXIDE CYCLASE* gene family, *AOC* expression, BiFC, jasmonate biosynthesis, organ-specific promoter activity, protein–protein interaction, redundancy.

Introduction

Jasmonates and octadecanoids are essential signals in plant responses to abiotic and biotic stresses as well as in plant development. Jasmonic acid (JA), its methyl ester (JAME), and its amino acid conjugates, altogether commonly named

jasmonates, as well as octadecanoids, which comprise *cis*-(+)-12-oxophytodienoic acid (OPDA) and its metabolites, originate from α-linolenic acid (α-LeA) of chloroplast membranes. Upon oxygenation by a 13-LIPOXYGENASE (13-LOX) an unstable allene oxide is formed by a 13-ALLENE OXIDE SYNTHASE (13-AOS) and subsequently cyclized by an ALLENE OXIDE CYCLASE (AOC) to *cis*-(+)-OPDA. The latter enzyme is of special importance in JA biosynthesis, since the specific enantiomeric structure of the naturally occurring (+)-7-*iso*-JA is established. Upon reduction of the cyclopentenone ring by OPDA REDUCTASE3 (OPR3) and three-times β-oxidative degradation of the carboxylic acid side chain, (+)-7-*iso*-JA is formed. Conjugation with isoleucine by JAR1 (Staswick and Tiryaki, 2004) leads to (+)-7-*iso*-JA-Ile, the most bioactive compound among jasmonates (Fonseca *et al*., 2009). The first three enzymes in JA biosynthesis, 13-LOX, 13-AOS, and AOC are located in plastids, whereas OPR3 occurs in peroxisomes, where the β-oxidation steps also take place. Genes encoding JA biosynthetic enzymes have been cloned from several plants and are often found to be encoded by gene families (for reviews see Wasternack, 2007; Browse, 2009*b*; Schaller and Stintzi, 2009).

In *Arabidopsis*, four genes code for 13-LOXs (Feussner and Wasternack, 2002), whereas the 13-AOS is encoded by a single gene (Laudert *et al*., 1996). Four genes, *AOC1* (At3g25770), *AOC2* (At3g25780), *AOC3* (At3g25760), and *AOC4* (At1g13280) code for functional AOC enzymes (Stenzel et al., 2003*b*) and five OPR encoding genes were identified (Schaller and Stintzi, 2009). The final steps in JA-biosynthesis, the β-oxidative shortening of the carboxylic acid side chain, are realized by enzymes of fatty-acid β-oxidation (e.g. ACX) which are also encoded by gene families in *Arabidopsis* and tomato (Castillo *et al*., 2004; Li *et al*., 2005; Theodoulou *et al*., 2005; Delker *et al*., 2007). The biosynthesis of jasmonates is assumed to be regulated primarily via substrate availability, a feed-forward regulatory loop, and by tissue-specific occurrence of the biosynthesis proteins (Wasternack, 2007). In addition, functional specification of individual members of the respective gene families offers another level of regulatory potential which has only been addressed marginally. Among the four 13-LOXs of *Arabidopsis*, LOX3 and LOX4 seem to function in JA formation required for proper anther development and thus, male fertility (Caldelari *et al*., 2011). By contrast, LOX2 is known to be involved in wound-induced JA generation and senescence-related jasmonate formation (Glauser *et al*., 2009; Seltmann *et al*., 2010). Among the five OPRs of *Arabidopsis* only OPR3 specifically converts *cis*-(+)-OPDA which can now be explained by the specificity of the binding pocket elucidated by the crystal structure of OPR3 (Breithaupt *et al*., 2006; Schaller and Stintzi, 2009). Among the individual members of gene families encoding enzymes of fatty acid β-oxidation including the required acyl-CoA ester formation, specific and partial redundant functions in JA-biosynthesis were observed (Schneider *et al*., 2005; Koo *et al*., 2006).

While AOC catalyses an undoubtedly crucial step in JA-biosynthesis, the question of specificity or redundancy in the function of the four *Arabidopsis* AOCs has not yet been addressed. It is largely unknown which and how the four AOCs contribute to JA biosynthesis *in vivo*. This may occur by spatial and temporal differences in expression, by different substrate specificities, and/or activity regulation. The crystallization of the *Arabidopsis* AOC2 has identified the protein as a member of the lipocalin gene family that forms trimers *in vitro* (Hofmann *et al*., 2006). This multimerization offers an additional level of regulation by potentially differential properties of AOC homo- and heteromers. Therefore, organ- and tissue specific expression of the *AOC* gene family members were analysed and putative heterodimerization among the four AOCs was inspected *in vivo*.

Organ-specific expression patterns were analysed by qRT-PCR, *in silico* analyses (www.genevestigator.ethz.ch), and by comparative analyses of promoter activities of all *AOC* gene family members in various organs and tissues during the development of *Arabidopsis*. The data revealed redundant and non-redundant promoter activities, which correspond to expression data and *in situ* hybridization. JA treatment increased the individual *AOC* promoter activities but general patterns were not altered. In most organs and tissues of untreated plants high AOC promoter activity correlated with known expression of JA-inducible genes. Heteromerization among different AOCs was observed *in vivo* which suggests another putative level of activity regulation in JA-biosynthesis.

Materials and methods

Enzymes and chemicals

Oligonucleotides were purchased from MWG Biotech (www.mwg-biotech.com), and restriction and DNA modifying enzymes were obtained from MBI Fermentas (www.fermentas.de). 5-Bromo-4-chloro-3-indolyl-β-D-gluconide cyclohexylammonium salt was purchased from Glycosynth (www.glycosynth.co.uk).

Plant material and treatment

Arabidopsis thaliana, ecotype Columbia (Col-0) was used throughout this study and cultivated in controlled chambers (Percival, CLF, Plant Climatics, www.plantclimatics.de) as described by Stenzel *et al*. (2003*b*). All seeds were surface-sterilized in 70% ethanol for 5 min, in 5% NaOCl/0.15% Tween 20 for 10 min, washed extensively in sterile distilled water and cold treated at 4 °C for 2 d before plating them on half-strength Murashige and Skoog medium containing 0.8% plant agar (Duchefa, www.duchefa.com) and 50 μg ml^{-1} kanamycin for the selection of transgenic plants. Seedlings were grown at 23 °C under a 16/8 h light/dark cycle. For any JAME-treatment the Murashige and Skoog medium was supplemented with 50 nM, 10 μM, and 100 μM JAME, respectively. *aoc1* (GABI KAT 845C10), *aoc3* (SALK101850), and *aoc4* (SALK124879) T-DNA loss of function mutants were obtained from GABI KAT and NASC.

Quantitative RT-PCR analysis of transcript accumulation

Total RNA was extracted from 50–100 mg tissue by the Qiagen RNeasy Mini Kit (www.qiagen.com) including an on-column DNase digestion. After quality control by gel electrophoresis, 3 μg of total RNA were used for first-strand cDNA synthesis by Superscript III reverse transcriptase (Invitrogen) following the manufacturer's instructions. Quantitative real-time RT-PCR was performed in an Mx3005P™ QPCR System (Stratagene, www.stratagene.com) using the Power SYBR® Green PCR Master Mix (Applied Biosystems, www.appliedbiosystems.com) and the primers are given in Supplementary Table S1 at *JXB* online. For each reaction, 20 ng of total cDNA was used as template for the generation of *AOC1*, *AOC2*, *AOC3*, and *AOC4* amplicons. The cDNA of *AtPP2A* (At1g13320) served as a constitutively expressed control as described by Czechowski *et al*. (2005).

All assays were performed with two technical replicates, and three biologically independent samples were used. ΔCt-values were calculated by subtracting Ct-values of the target gene from the Ct-value of the constitutively expressed *AtPP2A* gene. Comparative expression levels (CELs) were calculated as $2^{\Delta Ct}$.

Cloning of promoters of AOC1, AOC2, AOC3 *and* AOC4

DNA manipulations were performed as described by Sambrook *et al*. (1989). The promoter regions between about 2000 bp upstream of the ATG and the first exon of each *AOC* gene were isolated by PCR from *Arabidopsis* genomic DNA using the primers given in Supplementary Table S1 at *JXB* online. Primer sequences were designed for *AOC1* (At3g25770), *AOC2* (At3g25780), and *AOC3* (At3g25760) from the sequence of the clone TAC K13N2 (Acc. No AB028607) and for *AOC4* (At1g13280) from the sequence of the BAC clone T614 (Acc. No AC011810). The promoter fragments were subcloned into the vector pCR2.1-TOPO (Invitrogen, www.invitrogen.com). The resulting clones were designated as promAOC1-TOPO, promAOC2-TOPO, promAOC3-TOPO, and promAOC4-TOPO, respectively, and were checked by DNA sequencing. The promoter length of each *AOC* promoter is: 2006 bp (*AOC1*); 1867 bp (*AOC2*); 1425 bp (*AOC3*), and 1898 bp (*AOC4*), respectively.

Generation of promoter::GUS *constructs and generation of* promoter::GUS *lines*

The generation of the different *AOC::GUS* constructs is summarized in Supplementary Table S2 at *JXB* online. The correct transitions between *AOC* promoter sequences and the *GUS* gene were checked by PCR followed by sequencing. For PCR, a forward primer upstream of the multi cloning site of the pBI101.1 vector and the pBI101.3 vector and a reverse primer downstream of the initiation codon of the *GUS* gene were used (see Supplementary Table S1 at *JXB* online). The resulting *promoter::GUS* constructs have been transformed into *Agrobacterium tumefaciens* GV3101.

One-month-old *Arabidopsis* plants were transformed with *A. tumefaciens* carrying the corresponding promoter constructs by vacuum infiltration (Bechtold and Pelletier, 1998). Homozygous T_3 lines were generated originating from 10, 17, 12, and 18 T_1 lines transgenic for the promoter of *AOC1*, *AOC2*, *AOC3*, and *AOC4*, respectively, as well as the *GUS* reporter gene. Up to 30 individual plants of the T_3 generation of three independent homozygous lines for each construct were inspected.

BiFC assays in mesophyll protoplasts of Arabidopsis *and leaves of* N. benthamiana

For each AOC, the cDNA including the plastid transit sequence was fused at the 3'-end with the cDNA encoding either the C-terminal (YFP^C) or the N-terminal (YFP^N) half of YFP using pUC-SPYCE and pUC-SPYNE, respectively (Walter *et al*., 2004). From each construct 15 µg DNA was used for transient transformation of *A. thaliana* mesophyll protoplasts as described by Sheen (2002). For transient transformation of *N. benthamiana* leaves, constructs were transferred into *A. tumefaciens* strain GV 2260. Fully developed *N. benthamiana* leaves of 4-week-old plants grown in a greenhouse were injected into the lower side with a suspension of *A. tumefaciens* carrying the corresponding AOC:$YFP^{N/C}$ constructs or the empty vector as the negative control. Infiltrated plants were kept in a greenhouse for 5 d. The marked areas of infiltration were cut out and used for analysis by confocal laser scanning microscopy (CLSM, see below) and qRT-PCR as described above using *NtRPS6*, the tobacco gene encoding the ribosomal protein S6, as a constitutively expressed control and the primers listed in Supplementary Table S1 at *JXB* online.

Bimolecular fluorescence complementation was analysed by CLSM using a LSM510 META (Carl Zeiss, www.zeiss.de) using 514 nm for excitation of YFP and a lambda scan (516–700 nm) for recording YFP and chlorophyll fluorescence. Data evaluation was performed with the LSM Image Browser Software (Carl Zeiss). Relative fluorescence intensities were calculated using peak emission of YFP (527 nm) in a defined region of interest at constant pinhole size.

Histochemical GUS assay, in situ *hybridization, and immunolocalization*

Histochemical assays of GUS activity in transgenic lines were performed according to Jefferson *et al*. (1987). Seeds, seedlings, adult plants or their organs were vacuum-infiltrated with 100 mM sodium phosphate pH 7.0, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 10 mM EDTA, and 0.1% Triton X-100 containing 2 mM 5-bromo-4-chloro-3-indolyl-β-D-gluconide cyclohexyl ammonium salt. If not otherwise indicated, incubation was done at 37 °C for 18 h. Subsequently, the samples were transferred to 70% ethanol to remove chlorophyll. For cross-sections, stained leaves were embedded in paraplast (Sigma-Aldrich, www.sigmaaldrich.com), sectioned, deparaffinized, and rehydrated as described by Hause *et al*. (2003*b*). *In situ* hybridization with gene-specific probes for *AOC1*, *AOC2*, *AOC3*, and *AOC4* was performed as described by Maucher *et al*. (2000). Immunolocalization of AOC in cross-sections of *N. benthamiana* leaves was performed as described previously (Hause *et al*., 2000).

Results

Organ-specific mRNA accumulation of AOC *gene family members*

To inspect putative redundancy among the *AOCs* in terms of mRNA accumulation, transcript accumulation of all four genes was first analysed by qRT-PCR analysis in various organs and developmental stages of *A. thaliana* plants. Using primers specific for individual *AOC*-cDNAs, accumulation of transcripts of each of the four *AOC* genes was analysed in juvenile, adult, and old rosette leaves, stems, cauline leaves, flower buds, and open flowers, all from 10-week-old plants (Fig. 1A, 1B). Obviously, *AOC1* and *AOC2* expression was high in all leaves, whereas *AOC3* and *AOC4* were preferentially expressed in roots (Fig. 1C). This corresponds to *in silico* data (www.genevestigator.ethz.ch, Fig. 1D) with the exception that the expression of *AOC1* and *AOC2* cannot be detected individually by the ATH1 micro array as it lacks *AOC1*- and *AOC2*-specific probe sets. The *AOC1*-labelled probe set is ambiguous and putatively also detects other *AOC* transcripts, for example, *AOC2*. The transcript accumulation of all

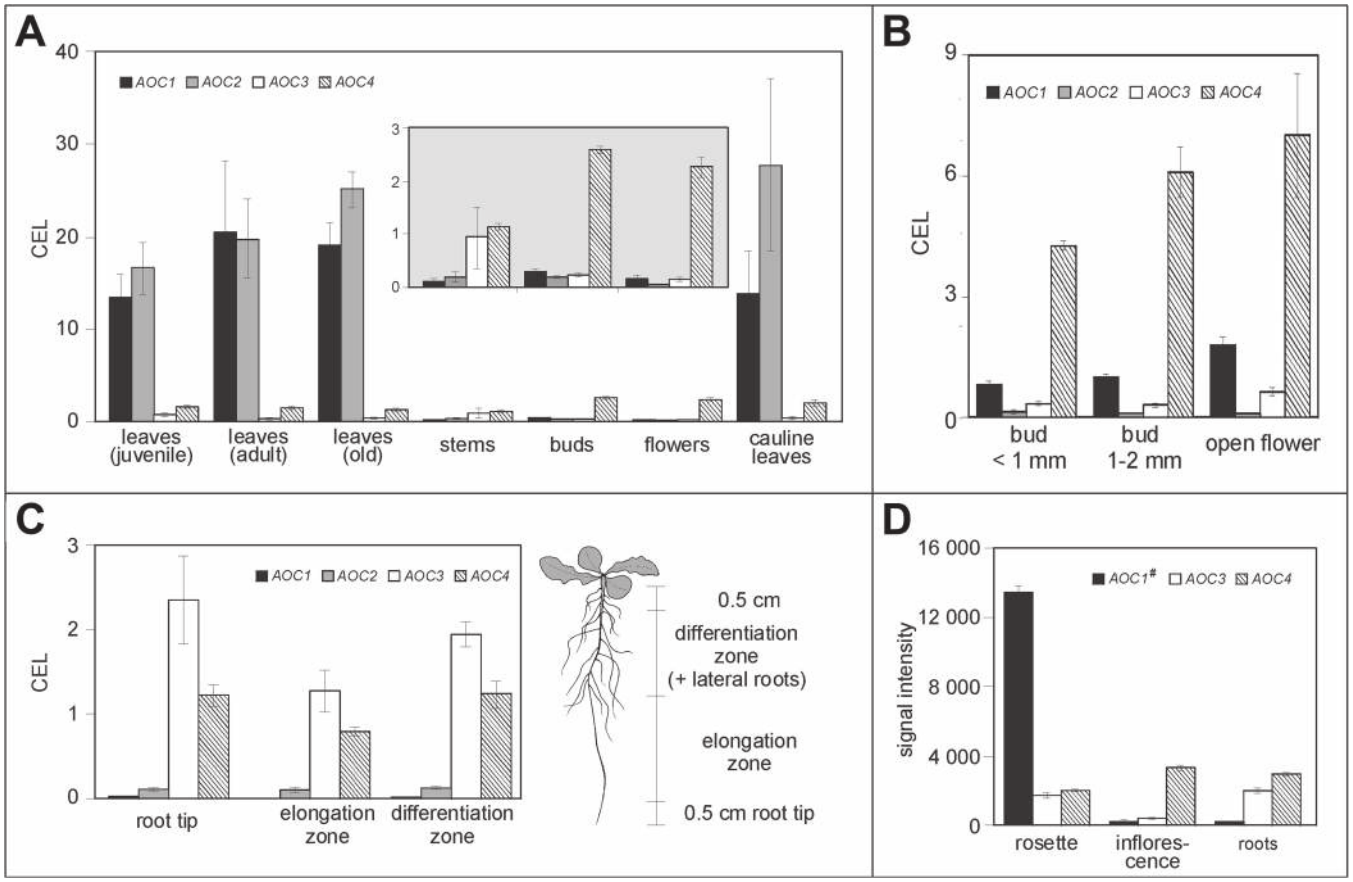

Fig. 1. Organ-specific expression analysis of *AOC* gene family members. Analyses were performed with different organs of 10-week-old plants grown under long-day conditions (A, B) or with roots of 10-d-old seedlings grown under continuous light (C). (A–C) qRT-PCR analyses of *AOC1–4* expression. *AtPP2A* (At1g13330) served as a constitutively expressed control gene. Data represent mean values and standard errors of three biological replicates. The inset in (A) shows a magnification of the expression in stems, buds, and flowers. Microarray data (D) of *AOC* expression in wild-type organs were taken from the gene atlas (www.genevestigator.ethz.ch) (# indicates ambiguous probe for *AOC1*, which may detect partially other *AOCs*). CEL, comparative expression level.

AOC genes in stems, flower buds, and open flowers is relatively low (Fig. 1A, inset, Fig. 1B), but a preferential accumulation of *AOC4* mRNA and, to a lesser extent, of *AOC1* mRNA in flowers points to a putative function of these AOCs in JA biosynthesis during flower development.

AOC promoter activities during seedling development

In order to elucidate tissue-specific expression of all four *AOC* genes and to confirm transcript data from various plant organs, promoter activities of *AOC1*, *AOC2*, *AOC3,* and *AOC4* were analysed during growth and development. The promoter region covering the region 1.4–2.0 kb upstream of the ATG and the first exon of each *AOC* gene was used to transform *A. thaliana* Col-0 plants with the respective *promoter::GUS* constructs (see Materials and methods for details). At least 10 independent T_1 lines were used to select three representative lines each, which were further cultivated to generate homozygous lines. Homozygous T_3 transgenic seeds were inspected prior to imbibition and 1, 2, and 3 d after germination (dag) (see Supplementary Fig. S1 at *JXB* online). Dry seeds were free of any *AOC* promoter activities, but *AOC4* promoter activity was already detectable in the root tip at 1 dag, and increased during the following 2 d. Promoter activity of *AOC1* appeared first at 2 dag in the cotyledons followed by *AOC2* promoter activity at 3 dag, whereas initial *AOC3* promoter activity was detectable in the hypocotyl and less in meristematic cells of the root tip at 3 dag. *AOC4* promoter activity appeared strongly in meristematic cells of the root tip, in the root cap, and less in the hypocotyl of seedlings at 3 dag.

In 1-week-old seedlings, most of the promoter activity patterns of seedlings at 3 dag were maintained, but at a higher level and were extended to the apical meristem in the case of *AOC1* and *AOC3* (Fig. 2). Highly localized promoter activities appeared for *AOC3* and *AOC4* in the root tip. The *AOC3* promoter was active only in meristematic cells of the root tip, whereas *AOC4* promoter activity was also visible in the root cap. Both promoters were active at the site of lateral root formation. In 2-week-old plants, strong promoter activities of *AOC1* and *AOC2* and weaker activity of the *AOC3* promoter throughout all leaves was found, whereas the *AOC4* promoter was highly active in the main vein of all leaves (Fig. 3A). Promoter activity patterns of *AOC3* and *AOC4* in the primary roots as well as the sites of lateral root out growth were largely consistent with those of 1-week-old plants, but intensities were stronger. In the case of

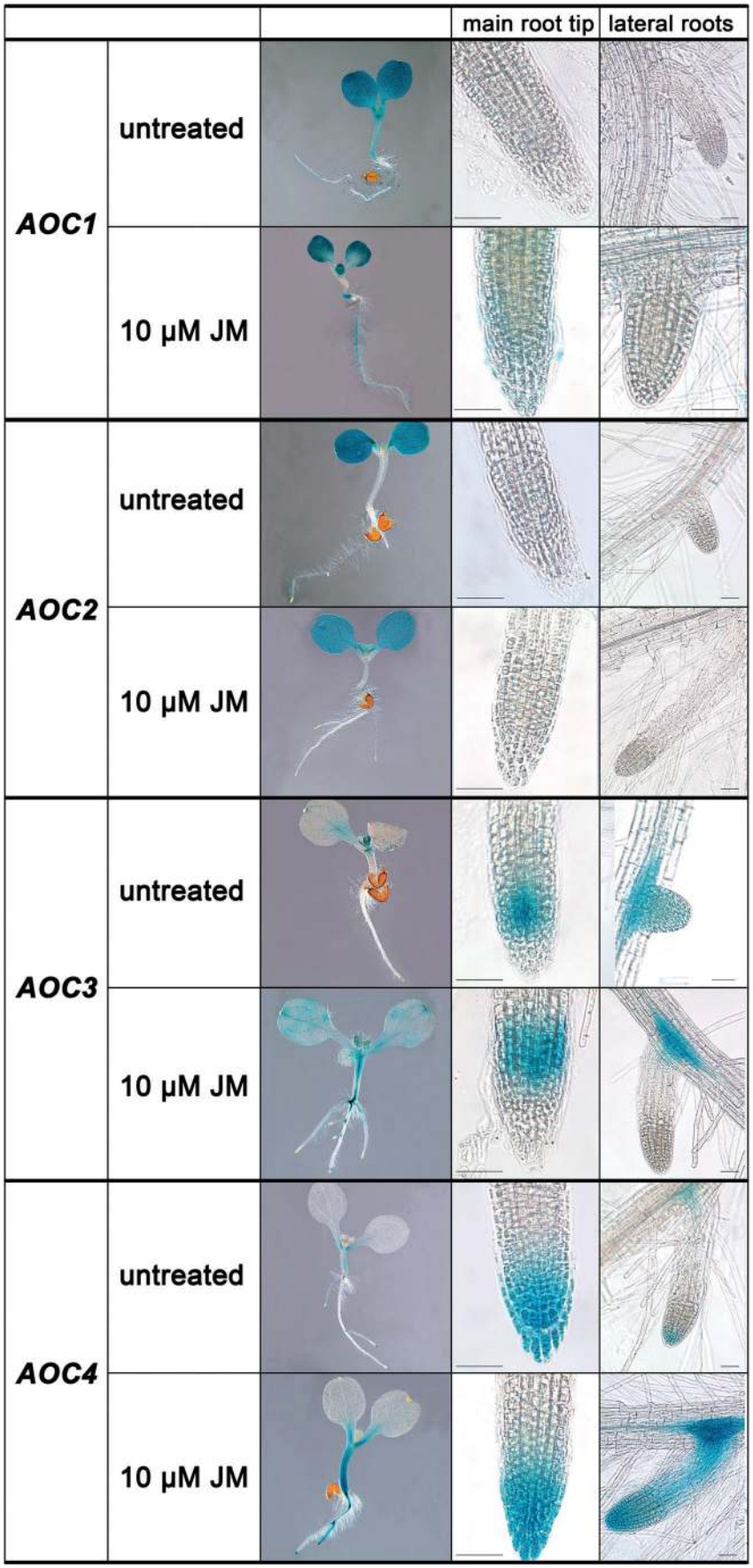

Fig. 2. Promoter activities of *AOC* gene family members in 1-week-old seedlings of the respective GUS-reporter lines non-treated or treated with 10 µM JAME (JM), and magnified views of primary root tips and lateral root tips. Bars represent 50 µm.

AOC3, a shift of the localized promoter activity was observed in meristematic cells of 1-week-old primary roots (Fig. 2) into the elongation zone of 2-week-old primary roots (Fig. 3D), whereas *AOC4* promoter activity was expanded to the central cylinder of the root elongation zone.

Promoter activities in fully developed plants

In 1-month-old plants promoters of *AOC1*, *AOC2*, and *AOC*3 were active in rosette leaves throughout the leaf area with slightly higher activity in vascular tissue, whereas the *AOC4* promoter activity was restricted to the major vein and first order minor veins, but did not appear in the veins where phloem loading takes place (Fig. 3E). Inspection of cross-sections confirmed this pattern and showed promoter activity for *AOC1* and *AOC2* in the epidermal layer as well (Fig. 3F). The specific *AOC4* promoter activity in vascular bundles observed in the leaf overview was also confirmed by cross-sections (Fig. 3F). In roots, the following activity patterns appeared: the *AOC4* promoter was preferentially active in the root tip of primary and secondary roots and in lateral root primordia, and the promoters of *AOC3* and *AOC4* were both active in the ramifications of primary and secondary roots (not shown). The promoter activity of the aboveground parts of 1-month-old plants persisted in 2-month-old plants, where *AOC1, AOC2*, and *AOC3* promoters were active. The *AOC4* promoter was also active in most cauline leaves and young leaves, but less active in senescent leaves and stems (see Supplementary Fig. S2 at *JXB* online).

Promoter activities in flowers and during embryo and seed development

Young flower buds exhibited strong activity of the *AOC1* promoter in sepals and petals, whereas very weak activity was observed for *AOC2, AOC3*, and *AOC4* in sepals (data not shown). Open flowers exhibited high activity of *AOC1* and *AOC4* promoters in sepals, petals, the transmission tissue of the pistil and in filaments of stamens (Fig. 4A, 4C, 4D). For *AOC2* and *AOC3*, only weak promoter activities were detected in sepals and for *AOC3* in the stigma. Detailed inspection of mature pollen and anthers revealed high activity of the *AOC1* promoter in anthers and released pollen, weak activity of the *AOC4* promoter in some pollen grains, and no promoter activity of *AOC2* and *AOC3* in anthers and released pollen (Fig. 4D, 4E). The GUS activity detected for the *AOC4* promoter in the flower stalk was confirmed by *in situ* hybridization with gene-specific probes (Fig. 4B). Expression of *AOC1*, *AOC2*, and *AOC3* could not be detected by *in situ* hybridizations in the flower stalk, thus corresponding to the lack of GUS activity. The promoter activity data in general correspond well with the *in silico* expression data for (*AOC1), AOC3*, and *AOC4* obtained

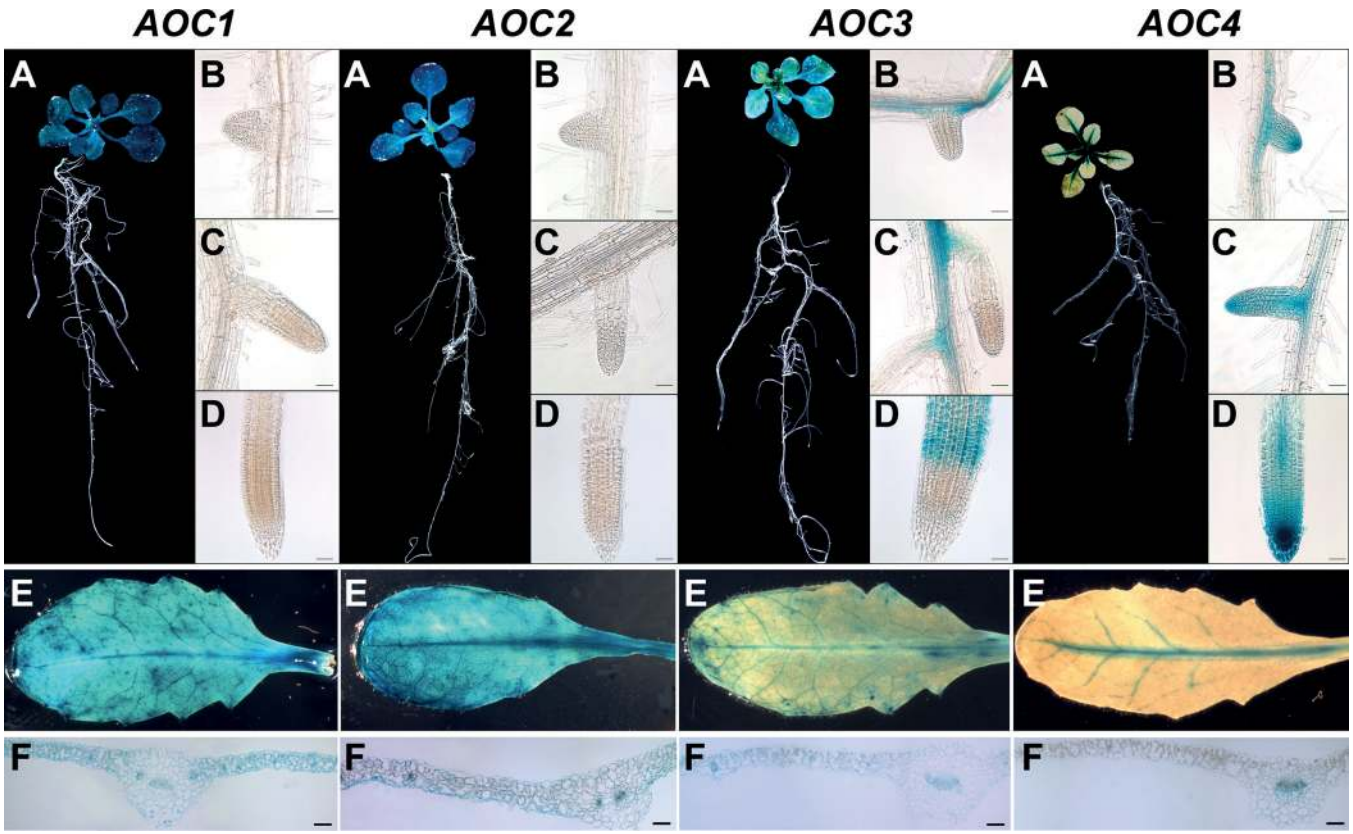

Fig. 3. Promoter activities of *AOC* gene family members in 2-week-old seedlings (A, D) and fully developed rosette leaves of 1-month-old plants (E, F) of the respective GUS-reporter lines. (A) Survey about the total seedlings from which magnifications (B), (C), and (D) were depicted. (B) Lateral root primordia. (C) Lateral roots. (D) Tip of primary root. (E) Top view and (F) cross-sections of rosette leaves. Bars represent 50 μm (B–D) and 100 μm (F).

from Genevestigator database (https://www.genevestigator.ethz.ch/; Zimmermann *et al*., 2005).

Inspection of embryo development did not reveal promoter activity of any *AOC* gene family member in fertilized ovules or during various stages of embryo development (see Supplementary Fig. S3 at *JXB* online). These data correspond to undetectable *AOC* promoter activities in mature seeds in fully developed siliques (see Supplementary Figs. S3E and S1 at *JXB* online). Strong activities, however, of *AOC3* and *AOC4* promoters were detected in the valves and the abscission zones of siliques.

Organ- and tissue-specific expression of the AOC *gene family members upon JA treatment*

Since *AOC* transcript accumulation in leaves has been shown to increase after JA treatment (Stenzel *et al*., 2003*b*), it was

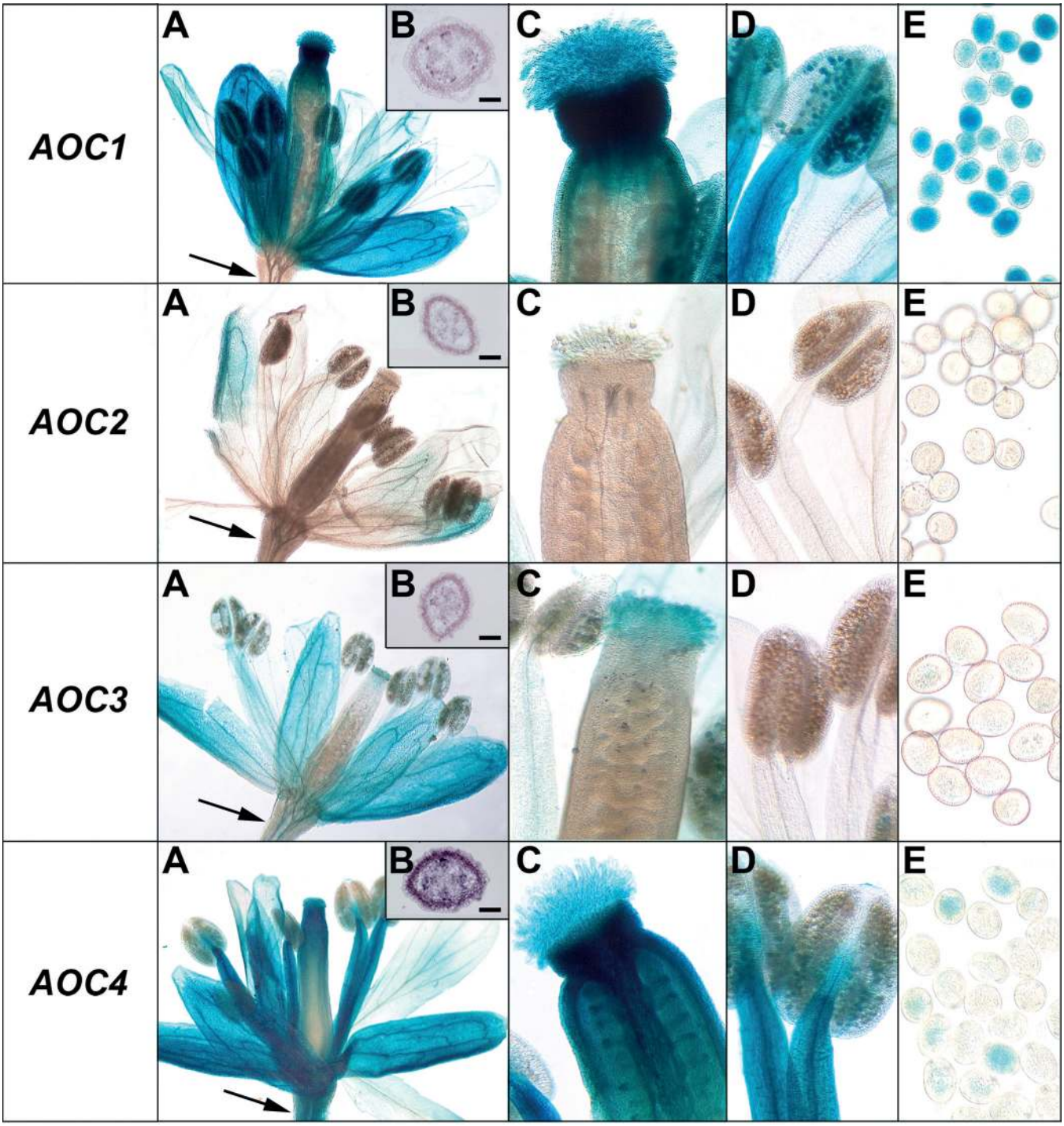

Fig. 4. Promoter activities of *AOC* gene family members in open flowers of two-month-old plants of the respective GUS-reporter lines. (A) Whole flower. (B) *In situ* hybridization with gene-specific probes in cross-sections of the flower stalk. (C) Stigma. (D) Anthers. (E) Pollen. Bars represent 50 μm (B). Arrows in (A) indicate the region used for cross-sections shown in (B).

analysed whether JA treatment alters organ- and tissue-specific promoter activities of *AOC1*, *AOC2*, *AOC3*, and *AOC4*. The corresponding transgenic lines were grown for 7 d on agar plates and were kept untreated (control) or were treated with 50 nM, 10 µM, and 100 µM JAME for 16 h. Compared with the GUS activity of the non-treated plants, the general patterns of *AOC* promoter activities were not altered by any JAME concentration, but an elevated GUS activity could be seen for each line and each tissue as shown for 10 µM JAME and the main root tip as well as the lateral root (Fig. 2). This increase in activity was also reflected in mRNA accumulation data published for total AOC mRNA and AOC1–AOC4 mRNA (Stenzel *et al*., 2003*b*). The only exception was an additional *AOC4* promoter activity in the primary root stele near lateral root branching as well as in the elongating lateral roots. The induction of *AOC* gene expression by JAME was also confirmed by qRT-PCR analysis of the leaves and roots, respectively. In 10-d-old seedlings the expression of all four *AOC*s was increased by application of 10 µM JAME (see Supplementary Fig. S4B, D at *JXB* online). Similar results were obtained in response to the local wounding of leaves or roots of the respective plant material (see Supplementary Fig. S4A, C at *JXB* online). The transcriptional induction was stronger in leaves with *AOC1* and *AOC2* being highly expressed. In roots, predominant expression of *AOC3* and *AOC4* was confirmed. However, the presence of an inducing stimulus, such as treatment with JAME and local wounding, also triggered the expression of *AOC1* and *AOC2* in roots which further corroborates the hypothesis of functional redundancies among the *AOC* gene family members. This suggests that promoter activities of the *AOC* gene family members are regulated mainly developmentally but can be increased by inducing signals such as JA.

Loss-of-function mutants suggest functional redundancy of AOCs in JA biosynthesis

T-DNA loss-of-function mutants with compromised expression of the respective AOCs (see Supplementary Fig. S5 at *JXB* online) were analysed to assess functional redundancy of *Arabidopsis* AOCs *in planta*. *aoc1*, *aoc3*, and *aoc4* single mutants did not show phenotypic alterations in JA-related phenotypes such as root growth, lateral root development, flower development, and fertility (see Supplementary Table S3 at *JXB* online). In response to mechanical wounding, *aoc1* and *aoc4* single mutants showed slight yet significantly reduced OPDA levels 1.5 h post wounding. However, the lack of an additive effect in the *aoc1aoc4* double mutant delimits the biological significance of these results. Unfortunately, no *AOC2* T-DNA-insertion line was available to study the effects of *AOC2* loss *in vivo* and the tandem repeat organization of *AOC1*, *AOC2*, and *AOC3* prevented the generation of higher order mutants that combine *aoc1* and *aoc3*. The available double mutants *aoc1aoc4* and *aoc3aoc4* also lacked JA-related phenotypes. Remarkably, even tissues that showed a certain degree of specificity in the *promoter::GUS* analyses (e.g. *AOC1* and *AOC4* in anthers, Fig. 4) failed to show developmental defects associated with JA-deficiency (e.g. male sterility) in the respective double mutants (see Supplementary Table S3 at *JXB* online). In conclusion, these analyses indicate a generally high level of functional redundancy among the four *Arabidopsis* AOCs.

Homo- and heteromerization of the four AOCs in vivo

Enzymatically active AOC2 is known to form a trimer *in vitro* (Hofmann *et al*., 2006). Therefore, heteromerization of the AOC proteins might be another level of regulation in JA biosynthesis since the spatial and temporal pattern of promoter activities of the four *AOCs* exhibited partially redundant properties.

In order to inspect putative homomeric and heteromeric interactions of the different AOCs *in vivo*, BiFC analyses were performed using mesophyll protoplasts of *A. thaliana* transiently transformed with pairwise combinations of fusions of AOC1–4 with the N- or C-terminal half of YFP, respectively (Fig. 5). As visible by YFP fluorescence, all combinations of AOCs (homomeric as well as heteromeric) are able to reconstitute YFP, but with different appearance and intensities. As depicted for the combination AOC4-YFP^N/AOC4-YFP^C (Fig. 5A), strong fluorescence signals appeared as dots in chloroplasts indicating homomerization of AOC4. Other homomeric combinations showed a more diffuse signal within the chloroplasts, such as AOC3-YFP^N/AOC3-YFP^C. Analogous differences in the appearance of YFP fluorescence were also visible in heteromeric interactions. Although all of them showed fluorescence signals in chloroplasts, they appeared as dots (e.g. AOC4-YFP^N/AOC1-YFP^C) or partially diffuse signals (e.g. AOC3-YFP^N/AOC1-YFP^C). Using the calculation of relative fluorescence intensities, the homomeric interaction of AOC4 was highest compared with that of AOC1, AOC2, and AOC3 (Fig. 5B). The heteromeric interactions of AOC1, 2, and 3 were strongest with AOC4 independently of the type of combination, whereas combinations with AOC1 always exhibited the lowest intensities (Fig. 5B).

False-positive signals may occur in isolated protoplasts due to the protoplast isolation procedure and/or by the endogenously occurring AOC protein which is known to be abundant in fully developed leaves of *A. thaliana* (Stenzel *et al*., 2003*b*). Therefore, the data were verified by transient transformation of *N. benthamiana* leaves. The *N. benthamiana* AOC is encoded by a single gene, and the protein localization is confined to vascular bundles as shown in Supplementary Fig. S6 at *JXB* online. Thus, the use of *N. benthamiana* mesophyll protoplast circumvents putative artefacts caused by interactions of the heterologous expressed AOCs with an endogenous, untagged AOC. Data obtained using the *N. benthamiana* system verified the results recorded with mesophyll protoplasts of *A. thaliana* (see Supplementary Fig. S7 at *JXB* online)

A final question is whether the heteromeric interaction among the AOC proteins affects AOC activity. Differential activities could result in different capacities of JA formation which, in turn, would result in differences in the expression of JA-responsive genes. Advantage was taken of the well-known positive feed-back loop in JA biosynthesis, where an increased JA level leads to an increase in *AOC* expression (Wasternack, 2007). Two pairs of AOCs exhibiting either weak (AOC1-YFP^N/AOC2-YFP^C) or strong (AOC3-YFP^N/AOC4-YFP^C) interaction in both transformation systems were selected and

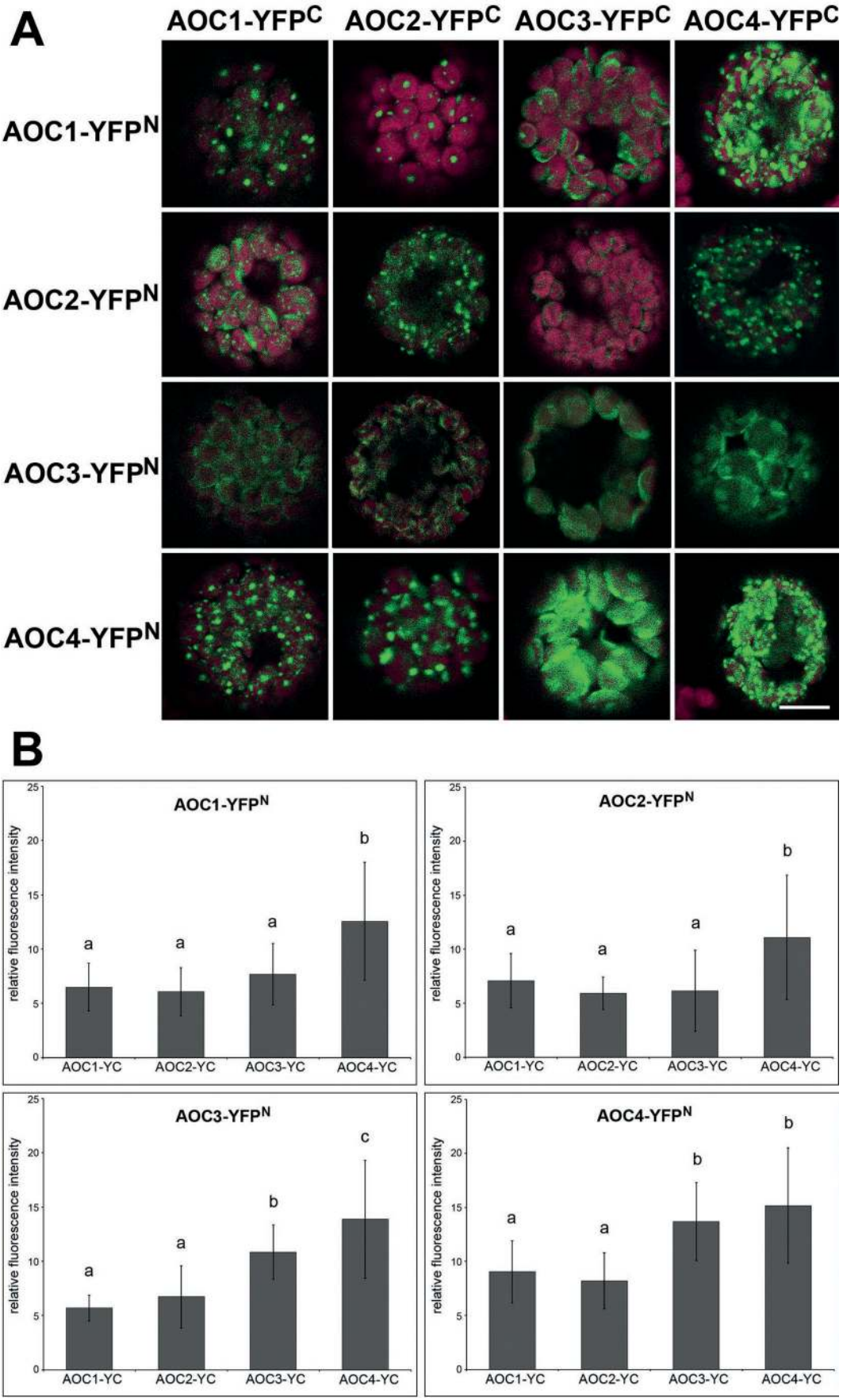

Fig. 5. Summary on BiFC analysis of AOCs in mesophyll protoplasts of *A. thaliana*. AOC1, 2, 3, and 4 were fused with either the N-terminal or the C-terminal half of YFP and pairwise expressed in mesophyll protoplasts. (A) YFP fluorescence recorded by LSM using

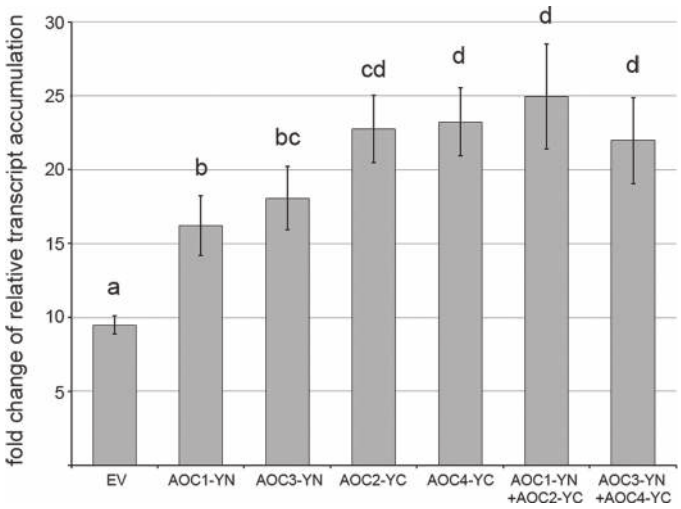

Fig. 6. Wound-induced transcript accumulation of *NbAOC* in leaves of *Nicotiana benthamiana* transiently transformed with various *AOC* constructs. AOC1, 2, 3, and 4 were fused with either the N-terminal or the C-terminal half of YFP and transiently expressed in leaves of *N. benthamiana* either alone or in two combinations (AOC1+AOC2 or AOC3+AOC4). Five days after infiltration, leaves were taken directly or wounded for 1 h and the expression of the endogenous *NbAOC* was recorded by qRT-PCR using *NtRPS6* as the constitutively expressed control. The mean of fold change ±SD (n=5) of wound-induced induction of *NbAOC* expression is shown. Different letters designate statistically different values (one-way ANOVA with Tukey's HSD test; $P \leq 0.05$).

expressed in *N. benthamiana* leaves. In both cases, YFP fluorescence was clearly visible confirming an appropriate expression level of the transgenes. Transcript accumulation of the internal *NbAOC* was recorded by qRT-PCR as a read-out of wound-induced JA formation in the respective transgenic tissues (Fig. 6). Compared with the empty vector control, there was a clear increase in *NbAOC* transcript accumulation by the transient transformation of constructs expressing individual *Arabidopsis* AOCs. However, upon heterologous expression of the two selected interaction pairs, *NbAOC* transcript accumulation was not changed significantly when compared with the expression of the individual *Arabidopsis* AOCs. Moreover, no correlation was visible depending on the strength of heteromeric interaction. This suggests that heterologous over-expression of *Arabidopsis* AOCs in *N. benthamiana* can be attributed to elevated JA levels leading to increased *NbAOC* transcript accumulation. Heteromerization of two distinct AOC pairs apparently did not alter the capacity of JA formation on a broad scale, yet a putative *in vivo* function might be masked by the over-expression in the heterologous system.

Discussion

Biosynthetic pathways of plants are often tightly regulated including gene families for important enzymatic steps. Such gene families allow a spatial and temporal fine-tuning in gene expression thereby sustaining the biosynthetic capacity at different levels. This is of preferential interest, if the preceding step is catalysed by an enzyme encoded by a single copy gene. In JA biosynthesis of *A. thaliana*, *AOS* is encoded by a single copy gene, whereas *AOC* genes represent a family of four members. This prompted us to extend our long-term effort to elucidate the regulation of JA biosynthesis by an analysis of expression and *in vivo* tests on putative heteromerization of the four AOCs as a level of AOC activity regulation.

In order to analyse the putative contribution of the *AOC* gene family members during development and in different organs, qRT-PCR analyses were performed initially. These analyses revealed a predominant expression of *AOC1* and *AOC2* in leafy organs, whereas *AOC3* and *AOC4* were expressed mainly in roots and flowers. Spatial and temporal patterns of promoter activities of *AOC–AOC4* were analysed with homozygous T_3 *promoter::GUS* lines. These GUS data imply redundant and non-redundant transcriptional activation of the *AOC* gene family members in specific tissues of various developmental stages and was verified by *in situ* hybridization for the flower stalk.

Promoter activities of AOCs in vegetative development

In case of cotyledons and fully developed leaves, strong promoter activities of *AOC1* and *AOC2* corresponded to the expression data (Fig. 1A) and to the abundant appearance of AOC protein (Stenzel *et al*., 2003*b*). This constitutively formed AOC may attribute to the rapid wound-induced JA formation within a few minutes, that is accompanied by transcriptional activation of JA biosynthesis genes (Stenzel *et al*., 2003*b*; Chung *et al*., 2008; Glauser *et al*., 2008; Koo *et al*., 2009). JA has a vital role in the response to wounding. This was recently demonstrated impressively by recording the highly indicative protein pattern of wounded *Arabidopsis* leaves (Gfeller *et al*., 2011). About 95% of wound-induced proteome changes were found to be de-regulated in the absence of JA.

The localized *AOC4* promoter activity in vascular bundles corresponds to the preferential *AOS* promoter activity in these tissues (Kubigsteltig *et al*., 1999). It is also reminiscent of the vascular bundle-specific *AOC* expression and JA formation in tomato leaves (Hause *et al*., 2000, 2003*a*; Stenzel *et al*., 2003*a*). Together with grafting experiments of wild-type and mutant tomato plants, the data point to a role of JA and its amino acid conjugate JA-Ile in systemic signalling in tomato (Koo and Howe, 2009). Grafting experiments with *Arabidopsis* plants as well as analyses of phloem exudates suggest a similar signalling function of JA/JA-Ile in *Arabidopsis* (Truman *et al*., 2007; Koo

a lambda scan, (B) relative fluorescence intensities for all interactions shown in (A). Mean and standard deviations ($n \geq 30$) is shown. Different letters designate statistically different values (one-way ANOVA with Tukey's HSD test; $P \leq 0.05$). Bar represents 5 μm for all micrographs in (A). Note that the level of red chlorophyll fluorescence depends on the presence of YFP due to the use of an excitation wave length of 514 nm followed by a lambda scan.

et al., 2009). Interestingly, *AOC* promoter activity in the vasculature of leaves coincides with promoter activity of *MYC4*, a new target of JAZ proteins involved in JA dependent regulation (Fernández-Calvo *et al*., 2011).

The observed redundant *AOC3* and *AOC4* promoter activities in root tissues coincide with the JA responsiveness of roots, for example, root tips exhibit an increased mitotic activity and formation of meristematic cell clusters following treatment with low (0.1–1 μM) JA concentrations (Capitani *et al*., 2005). Furthermore, high AOC promoter activity and high JA level were detected in the root tips of tomato (Stenzel *et al*., 2008). The high promoter activities of *AOC3* and *AOC4* in lateral root primordia correspond with the lateral root-promoting effect of jasmonates (Wang *et al*., 2002) and coincide with a high expression of *LOX6*, *AOS*, *AOC3, OPR3*, and *MYC2* in this tissue (www.arexdb.org). *MYC2* is JA-inducible, encodes a transcription factor active in the expression of many JA-responsive genes (Lorenzo and Solano, 2005), and is a positive regulator of lateral root formation (Yadav *et al*., 2005). Its expression is transiently up-regulated by JA in a COI1-JAZ-dependent manner (Lorenzo *et al*., 2004; Dombrecht *et al*., 2007).

It should be stressed, however, that, until now, tissue-specific promoter activities and expression of the *AOC* gene family members can give only hints on the capacity to form JA. None of the *promoter::GUS* lines available for JA-responsive genes exhibit the property of a cell- and tissue-specific indicator of JA levels as developed for auxin with the synthetic *DR5*- and the *BA3*-promoter fused to the reporter gene *GUS* (Ulmasov *et al*., 1997; Oono *et al*., 1998). More recently, an immuno-cytochemical approach was established based on JA/JA-Ile-specific antibodies for cell- and tissue-specific detection of these compounds in tomato and *Arabidopsis* (Mielke *et al*., 2011). These data reflect the *AOC* promoter activities detected here at least for leaves, thereby supporting the initial assumption on a tight link between *AOC* promoter activity and JA formation.

Promoter activities of AOCs *in generative development*

The characteristic pattern of promoter activities of the *AOC* gene family members in flower organs suggest an organ-specific JA generation as observed for tomato flowers (Hause *et al*., 2000). This is supported by the expression pattern of JA-responsive genes deduced from publicly available expression data such as Genevestigator (https://www.genevestigator.ethz.ch/) and by activity data for JA-responsive promoters (cf. below). The *AOC1* and *AOC4* promoters are highly active in filaments suggesting a link to the phenotype of mutants affected in JA biosynthesis and JA signalling such as *fad3fad7fad8*, *dad1*, *opr3*, and *coi1*. All these mutants exhibit short filaments which lead to male sterility (Browse, 2009*a*, *b*). In the case of JA biosynthesis mutants this phenotype can be rescued by JA. The role of JA in stamen development and filament elongation is also substantiated by direct measurement of JA in flowers of *dad1*. This mutant is affected in a phospholipase A1, the enzyme responsible for the generation of the JA substrate α-LeA (Ishiguro *et al*., 2001). The putative link between JA biosynthesis, DAD1 and flower development/stamen maturation is strongly supported by the fact that the floral homeotic gene *AGAMOUS* responsible for stamen development directly regulates transcription of *DAD1*, thereby affecting JA-dependent gene expression (Ito *et al*., 2007).

The obvious role of JA in filament elongation is linked with auxin. The mutant affected in the auxin response factors ARF6 and ARF8 (*arf6-2arf8-3*) exhibits short stamen filaments and undehisced anthers, is down-regulated in expression of JA biosynthesis genes, and exhibits lower JA levels in flower buds compared with the wild type (Nagpal *et al*., 2005). Interestingly, the *AOC1* promoter contains the canonical auxin response element (AuxRE, TGTCTC) within its first 100 bp (ATHENA database; O'Connor *et al*., 2005). The AuxRE can serve as an ARF-binding site that might putatively be targeted by ARF6 and ARF8. This putative cross-talk between auxin and JA signalling during flower maturation has been recently characterized by ARF6- and ARF8-induced JA biosynthesis and JA-induced expression of transcription factors MYB21 and MYB24 which promote petal and stamen development (Reeves *et al*., 2012).

AOC promoter activity in other flower organs (e.g. pollen, sepals, and stigmas) might reflect a general 'defence alert' status in the respective tissues to protect the generative tissues. This is supported by a corresponding high expression of JA-responsive genes such as *Thi2.1* and *AQUAPORIN2* (Kaldenhoff *et al*., 1995; Vignutelli *et al*., 1998) and those coding for plant DEFENSINs and VSP2 (Utsugi *et al*., 1998; Thomma *et al*., 2002).

In *Arabidopsis* flowers, JA deficiency preferentially affects male fertility whereas, in tomato flowers, the *COI1* homologue is linked to ovule development, and the *jai1* mutant affected in the *COI1* gene is female sterile (Li *et al*., 2004). These plant species-specific differences of JA signalling in flowers also occur with respect to *AOC* expression. In tomato, the single copy gene *AOC* is specifically expressed in ovules (Hause *et al*., 2000; Stenzel *et al*., 2008) whereas, in *Arabidopsis*, none of the four *AOCs* exhibited promoter activities in ovules and only weak activity of the *AOC4* promoter in the carpel wall of the ovaries (Fig. 4). Promoter activities of *AOCs* could not be detected in the embryo development of *Arabidopsis* (see Supplementary Fig. S3 at *JXB* online) thereby clearly differing from tomato (Goetz *et al*., 2012). The promoter activities observed for *AOC3* and *AOC4* in the valves of siliques might be linked to a role of JA in the partitioning of nutrients which is known to be a JA-dependent process (Rossato *et al*., 2002; Armengaud *et al*., 2004; Babst *et al*., 2005).

Some of the observed expression patterns point to a specific role of *AOC* gene pairs in distinct organs and developmental stages. Yet, the lack of any obvious JA-related phenotype in the respective loss-of-function mutants (e.g. male sterility) rather argues for a high degree of redundancy among the AOC family members.

Regulation of JA formation by heteromerization of AOCs

The redundant and non-redundant *AOC* expression patterns indicate that differential AOC presence/functions might contribute to a spatial and temporal fine-tuning of JA-dependent processes. Homo- and heteromeric interactions among AOCs could constitute an essential level of regulation in JA biosynthesis similarly to the situation found for the ethylene biosynthesis enzyme 1-aminocyclopropane-1-carboxylate synthase (ACS), which

is encoded by a gene family of nine members (Tsuchisaka and Theologis, 2004*a*, *b*). The AOC was initially purified from corn as a homodimer (Ziegler *et al*., 1997), but the crystal structure of AOC2 of *Arabidopsis* suggests that this AOC occurs as a trimer (Hofmann *et al*., 2006).

In vivo analyses were performed using BiFC in mesophyll protoplasts of *Arabidopsis*. Homomerization as well as heteromeric interactions were observed for all four AOCs (Fig. 5) suggesting heteromerization as another putative level of regulation of JA biosynthesis. However, transient over-expression of *AtAOC* pairs in *N. benthamiana* leaves was not accompanied by an altered wound-induced transcript accumulation of the JA-responsive *NbAOC* gene (Fig. 6). These results do not exclude unequivocally a regulation of the JA response by an AOC activity control via *in vivo*-occurring heteromerization of AOCs. It has to be assumed that the type of multimer formation depends on the differential endogenous levels of available AOC proteins, which differ remarkably as indicated by the expression and promoter GUS analyses. The full spectrum of activities in JA formation might only be visible under natural protein levels. Thus, the high over-expression upon control of the constitutive 35S promoter could mask potential effects by artificial high levels of proteins. Furthermore, the transient over-expression system does not allow the exclusive formation of heteromers to be controlled *in planta*. Homomers and heteromers could be formed simultaneously, leading to an overall high level of AOC protein and thereby overriding the putative effect of activity control. Nevertheless, the BiFC-based data shown here suggest multiple heteromerization among all AOCs. Such an interaction among the AOCs of *A. thaliana* is also reflected in the *Arabidopsis* interactome map (Consortium, 2011).

Taken together, the results showed a high level of redundancy among the *Arabidopsis* AOCs which might result in a putative homo- and heteromer formation among the four AOCs. Indeed, homo- and heteromer formation occurs *in vivo* as identified by BiFC analysis. This indicates the high potential of a regulatory function of this gene family at the protein level resulting in the plant's capacity to regulate JA formation by enzyme activity control via protein–protein interaction of AOCs. Even the final proof *in planta* is obviously masked by the over-expression conditions, and the data suggest a fine-tuning in JA formation during development and in response to various stimuli.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Fig. S1. Promoter activities of *AOC* gene family members in dry seeds and seedlings of the respective GUS-reporter lines grown in light for the indicated periods; dag, days after germination.

Supplementary Fig. S2. Promoter activity of *AOC* gene family members in 2-month-old light-grown plants.

Supplementary Fig. S3. Promoter activities of *AOC* gene family members in different stages of embryo and seed development as well as in mature siliques.

Supplementary Fig. S4. qRT-PCR analyses of wound- and JAME-induced induction of *AOC* gene expression.

Supplementary Fig. S5. qRT-PCR analysis of *AOC* expression in T-DNA insertion mutants.

Supplementary Fig. S6. Immunolocalization of AOC in leaves of *N. benthamiana*.

Supplementary Fig. S7. Summary on BiFC analysis of AOCs in leaves of *N. benthamiana*.

Supplementary Table S1. Summary of PCR primer sequences.

Supplementary Table S2. Plasmids and restriction enzymes used for generation of *promoter::GUS* constructs.

Supplementary Table S3. Phenotypes of single and double AOC loss-of-function mutants.

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