Poplar Allene Oxide Synthase 1 Gene Promoter Drives Rapid and Localized Expression by Wounding

Bin Lei a, Christopher J. Frost b, Tao Xu a, Joshua R. Herr c,d, John E. Carlson e and Haiying Liang a*

a Department of Genetics and Biochemistry, Clemson University, USA.
b BIOS Institute, University of Arizona, USA.
c Department of Plant Pathology, University of Nebraska, Lincoln, NE, USA.
d The Center for Plant Science Innovation, University of Nebraska, Lincoln, NE, USA.
e The Schatz Center for Tree Molecular Genetics, Department of Ecosystem Science and Management, The Pennsylvania State University, University Park, PA, USA.

Authors’ contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

ABSTRACT

Promoters play critical roles in controlling the transcription of genes and are important as tools to drive heterologous expression for biotechnological applications. In addition to core transcription factor-binding motifs that assist in the binding of RNA polymerases, there are specific nucleotide sequences in a promoter region to allow regulation of gene expression. The allene oxide synthase (AOS) gene family are cytochrome P450s that are responsive to a variety of environmental stress, making them good candidates for the discovery of inducible promoters. *Populus* AOS homologs separate phylogenetically into two clades. Based on the 19 promoter motifs with significant abundance differences between the two clades, Clade I AOS genes are likely more responsive to hormones, salt, and pathogen, whereas clade II homologs are likely inducible by water stress. In this study, an upstream promoter from a Clade I poplar AOS encoding gene (AOS1) was cloned and used to drive the expression of a β-glucuronidase (GUS) gene in *Arabidopsis*. AOS is an
essentially enzyme in the lipoxygenase pathway that is responsible for the production of many non-volatile oxylipins in plants, including the jasmonates, which are regulatory phytohormones coordinating a variety of biological and stress response functions. Consistent with AOS transcript expression patterns, we found that the poplar AOS1 promoter drives rapid and localized expression by wounding. The study provides insight on the responsive elements in the poplar AOS promoters, but more importantly identifies a strong wound-inducible and localized promoter for future applications.

**Key Message**
- *Populus* AOSs separate phylogenetically into two clades, which show significant abundance differences in 19 promoter motifs.
- AOS1 is predominantly expressed in growing vascular tissue in *Populus*
- *Populus* AOS1 promoter drives rapid and localized expression by wounding in *Arabidopsis*.

**ABBREVIATIONS**
- AOS: Allene oxide synthase
- GUS: β-glucuronidase
- JA: Jasmonic acid
- MeJa: Methyl jasmonate

**1. INTRODUCTION**

Allene oxide synthase (AOS; hydroperoxide dehydratase; EC 4.2.1.92) is the first enzyme in the lipoxygenase pathway that is responsible for the production of many non-volatile oxylipins in plants, including jasmonates (jasmonic acid (JA) and methyl jasmonates (MeJAs)) [1]. Jasmonates play central roles in plant development and adaptations to both biotic and abiotic stresses. Likewise, AOS plays a similar role in regulating growth, development, flowering and adaptation to stresses. Not surprisingly, the AOS family proteins and their coding genes have gained much attention. AOS proteins and genes have been isolated and functionally characterized in several plant species, such as *Lycopersicon esculentum* [2], *Arabidopsis thaliana* [3], *Hordeum vulgare* [4], *Camellia sinensis* [5] and *Parthenium argentatum* [6]. AOS belongs to the cytochrome P450 family (CYP74A) and contains all four conserved domains characteristic of cytochrome P450 proteins [7].

AOS is a single gene in the *A. thaliana* genome (*AtAOS*). It is predominantly expressed in leaf and flower, with limited expression in root, stem and silique [3]. Under mechanical wounding, bothAtAOS mRNA and protein levels increased in wounded and systemic leaves [3], mirroring the β-glucuronidase (GUS) expression pattern when the scorable marker gene was driven by the AOS promoter. The studies of Laudert et al. [3] and Kubigsteltig et al. [8] found that jasmonates, such as 12-oxophytodienoic acid, octadecanoid analog (coronatine), and jasmonic acid could induce AtAOS expression locally. A microarray analysis of two *Arabidopsis* ecotypes revealed that induction of AOS transcripts by selenium was more pronounced in the resistant ecotype than in the susceptible one [9]. More recently, Naor et al. [10] reported that the AtAOS promoter activity was associated with feeding site and gall induction following nematode infection.

In flax (*Linum usitatissimum*), AOS was found in large quantities in the achenes [11], while in tomato AOS transcripts were detected only in the root [12]. Haga and Iino [13] reported four OsAOS gene homologs in the *japonica* rice genome. Both OsAOS1 and OsAOS4 were up-regulated by red and far-red light in seedling shoots. However, the response in OsAOS1 transcript occurred rapidly and transiently, while the response in OsAOS4 transcripts was slower and more sustainable. Furthermore, the maximal enhancement was greater in OsAOS1 transcripts than in OsAOS4 transcripts. Both OsAOS1 and OsAOS2 are wound inducible with induction being transient in OsAOS1 and sustainable in OsAOS2 (up to 2 days) [13-14]. In the study by Gnanaprakash et al. [15], a downy mildew fungus was found to significantly enhance pearl millet AOS1 expression in a resistant cultivar.

In woody plants, AOS expression can be inducible and organ-dependent. For example, transcript expression of AOS1 in poplar was strongly upregulated in leaf in response to insect herbivory [16]. Transcripts of a passion fruit (*Passiflora f. edulis flavicarpa*) AOS became detectable after mechanical injury and MeJA treatment, with both local and systemic induction and peaking at 9 h after wounding [17]. A peach
AOS1 transcript was detected in early fruit development and induced by MeJA and ibuprofen in the mesocarp tissue [18]. Similarly, the sole AOS in grapevine (Vitis vinifera) had highest expression in mesocarp tissue [19]. In addition, cacao and chestnut AOSs are pathogen-induced in resistant cultivar/species [20-21], and the trifoliate orange (Poncirus trifoliata) homolog was found up-regulated under drought.

Variation in AOS expression patterns among and within species, and among tissues and treatments, suggests that sequence differences in promoter regions be investigated, which might reveal novel promoter regions or motifs. The characterization of expression profiles of AOS promoters has been reported only in Arabidopsis [8-10], soybean [22] and trifoliate orange [23]. In the current study, we analyzed the sequence of AOS1 in Populus and investigated its promoter activity under wounding. Populus is an economically and ecologically important genus and model system for tree research. Poplars are native to the Northern Hemisphere and are among the fastest-growing temperate trees. Lawrence et al. (2006) reported ~ 9-fold and 26-fold changes, respectively, in transcript levels of a P. trichocarpa AOS gene in response to gypsy moth feeding and mechanical wounding. Frost et al. [16] observed similar expression changes of AOS1 in the hybrid poplar clone, OGY (P. deltoides x P. nigra) following gypsy moth feeding. Infection by leaf rust fungi Melampsora spp. also enhanced AOS expression in a fold change range of 1.8 to 3.2 [24]. These studies suggest that the Populus AOS1 promoter is strongly inducible to biotic stress and therefore a good candidate as a novel inducible promoter. In the current study, we further investigate AOS structure and functions in Populus, contributing to the understanding of this strong wound-inducible and localized promoter for future applications.

2. MATERIALS AND METHODS

2.1 Cloning of Poplar Hybrid OGY AOS1 Gene and Protein Alignment

Hybrid poplar OGY plants were propagated by rooted cuttings and grown in a greenhouse under a 14/10 h photoperiod and a temperature range of 22 to 25°C. One hour after being crushed with a pair of pliers, wounded leaves were harvested, immediately frozen in liquid nitrogen, and then stored in an ultra-low freezer until being processed for RNA extraction using a QIAGEN RNeasy Plant Mini Kit (QIAGEN, CA). RNA quality and quantity were evaluated with a denaturing agarose gel stained with ethidium bromide. cDNAs were synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA) and utilized as the cloning template. Based on the sequence and annotation available for the Populus trichocarpa genome (Phytozome, version 1.1), we designed primers (5’AACATGGCTTCCTCTCC3’ and 5’CTCGGAAAGCATTTGGTAA3’) for the amplification of the AOS1 coding sequence, including four nucleotides before the start codon and 103 nucleotides after the stop codon. After sequencing, the deduced poplar AOS1 protein sequence was aligned with homologous sequences from Populus trichocarpa (Potri.002G130700.1, Potri.014G038700.1, Potri.009G109700.1, Potri.004G149000.1, Potri.004G148900.1, Potri.004G148600.1, Phytozome, version 4.1), Populus deltoides (Podel.14G039700.1, Podel.02G143700.1, Podel.04G152600.1, Podel.04G152500.1, and Podel.09G112300.1, Phytozome, version 2.1), Glycine max (NP_001236445.1), Arabidopsis thaliana (Y12636), Linum usitatissimum (U00428), Hordeum vulgare (AJ250864 and AJ251304), Oryza sativa Japonica (XP_015631686.1), Prunus persica (XP_007222520.1), Medicago truncatula (XP_013466038.1), Castanea mollissima (KAF3975091.1), Taxus chinensis (ATG29971.1), Picea sitchensis (ABK25164.1), Pohlia nutans (QCF46585.1), Amborella trichopoda (XP_006856192.1) with Multalin [25]. Default parameters were applied. TargetP-2.0 was employed to predict the presence of N-terminal sequences (http://www.cbs.dtu.dk/services/TargetP/), while Phyre2 (http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index) was used for prediction of secondary structure. The phylogenetic tree for AOS proteins was constructed with the MEGA X software using the neighbor-joining method. Bootstrap tests were performed with 1,000 replicates for statistical reliability.

2.2 Detection of OGY AOS1 Expression with Reverse Transcription-quantitative Polymerase Reaction (RT-qPCR)

Leaf, petiole, phloem, and xylem tissues were collected from cuttings of the OGY hybrid propagated in a growth chamber. Uniform in size, the poplar cuttings were maintained in a walk-in
growth chamber at 25°C with a 16/8 h photoperiod. Cuttings were planted in 5-gallon pots with a commercial potting soil (MetroMix 250, SunGro, Bellevue, WA, USA) and watered as necessary. Samples from three individual cuttings were harvested when the plant height reached approximately 1.5 m tall and served as biological replicates. Leaf samples were harvested from mature tissue – leaf plastochron index (LPI) 15 was used in this experiment, and the leaf blade was excised from the petiole. A region of stem from LPI 14-16 was harvested and the phloem and xylem material were separately and carefully scraped into a collection container made of aluminum foil. All four tissue types were immediately flash frozen in liquid nitrogen and immediately stored at -80°C until processed. RNA was extracted using a modified CTAB method [26] and real-time qPCR was performed using ribosomal 18S (forward: 5′ATTGTTGTGCTTTCAACGAAG3′, reverse: 5′AADDCCAGGGACGTAGTCAA3′) as the housekeeping gene. Protocols for the real-time qPCR have been previously reported [16].

2.3 Conserved Motif Mining of Populus AOS Promoters, Construction of OGY AOS1 Promoter::GUS Binary Vector, and Arabidopsis Transformation

An approximately 2-kb promoter region upstream of the AOS1 start codon was cloned by genome walking using the genome of the hybrid poplar clone, OGY. Conserved motifs were analyzed using a database of Plant Cis-acting Regulatory DNA Elements (PLACE) [27] and then cloned into a pCAMBIA1391xa vector placed within BamHI and HindIII restriction enzyme sites to drive the expression of a β-glucuronidase gene from Escherichia coli (gusA). After sequence validation, the recombinant pCAMBIA1391 plasmid was introduced into Agrobacterium tumefaciens strain GV3101 by electroporation. Plant transformation was conducted in A. thaliana Col-0 via the floral-dip method according to Desfeux et al. [28]. Transgenic plants were selected by germinating Arabidopsis seeds in Perter's medium supplemented with 25 mg/L hygromycin as described in Xu et al. [29]. Transformation was verified by PCR with primers annealing to the AOS1 promoter (5′GAAAGCCATGGTGGGATT3′ and 5′GGAAAACAAATGGGGAAA3′) and the GUS gene (5′TGTGTCTATGATGATATGAT3′ and 5′CCAAAGCCAGTAAAGTAG3′). AOS promoter regions (2 kb in length) retrieved from the genome of P. trichocarpa (Phytozome, version 4.1) and P. deltoides (Phytozome, version 2.1) were analyzed as described above for OGY AOS1. The two tailed Student's t-test was employed to compare motif abundance differences between the two clades identified by the phylogenetic analysis. We utilized a P-value cutoff of 0.05 in the statistical tests.

2.4 GUS Staining and Quantification

T3 AOS1 promoter::GUS transgenic and non-transformed Arabidopsis plants were grown in a greenhouse under 20/15°C (day/night) with an 18/6 h photoperiod. Wounding was made by placing serrated forceps tips over and underneath a leaf specimen across midvein and then applying pressure. Leaves were collected for GUS staining before wounding (0 h), or 0.5, 1, 2, 6, 24 h after wounding. Unwounded leaves from the same plants were also collected at the time points. Stems, roots, and flowers were stained immediately after collection. Overnight GUS staining with X-Gluc was carried out according to Jefferson et al. [30]. All photos were taken under a Meiji Techno MX4300L dissection scope (Meiji Techno, CA). Quantification of GUS activity was conducted with a fluorometric essay with leaves of five transgenic lines 1 h after wounding. Total proteins were extracted from samples, and 4-methylumbelliferyl β-D-glucuronide (4-MUG) was used as a substrate as described by Jefferson et al. [30]. Hydrolyzed MUG product, 4-methylumbelliferone (MU), was detected, and GUS activity was expressed as nmols MU/min/mg total protein. Five biological replicates were included in the staining and fluorometric essays. Significance of differences between wounded and unwounded samples were examined with the paired Student's t-test, which was performed using SPSS version 17.0 (IBM Corp., Armonk, NY). The P-value cutoff was 0.01.

3. RESULTS

3.1 Cloning and Characterization of Full Length cDNA of OGY AOS1

The cDNA sequence of 1,765 bp contained an open reading frame of 1,578 bp, encoding a polypeptide of 526 amino acids with a calculated molecular mass of 59.0 kDa and an isoelectric point of 9.0. At the DNA level, the OGY AOS1 sequence shares an identity of 99.2%, 98.3%, and 97.9% to P. trichocarpa, P. alba, and P.
euphratica, respectively. Comparison with published sequences indicates that AOS is conserved across the plant kingdom (Supplemental Fig. 1). The hybrid poplar sequence showed 100%, 76.7%, 66.0%, 57.4%, and 51.2% identity with AOS1 in P. trichocarpa, Prunus persica, A. thaliana, Amborella trichopoda, and Picea sitchensis, respectively. The predicted protein secondary structure of OGY AOS1 is similar to that of the Arabidopsis homolog, having two binding sites for substrate and one for metal (heme axial ligand). OGY AOS1 contains a putative chloroplast transit peptide (likelihood=0.9997) and the four conserved domains characteristic of cytochrome P450 proteins that are common for all proteins in the AOS family are underlined in Supplemental Fig. 1. The consensus sequence P$\text{NKQCAG}$ of the heme binding domain and the highly conserved motif $\text{DGGKIL}$ of the CYP74A enzymes were found in OGY AOS1.

Six AOS homologs were identified in the P. trichocarpa genome when using A@OS sequence as the query. In P. deltoides, five copies were found. OGY AOS1 is most similar to gene models Podel.02G143700.1 and Potri.002G130700 (Fig. 1). The Potri.014G038700.1 and Podel.14G039700.1 AOS1 proteins form a sister group to OGY AOS1 and the aforementioned Populus sequences. All these sequences are in the same clade with A@OS and OsAOS1 (Clade I). The other Populus sequences are in a separate clade with OsAOS2, OsAOS3, OsAOS4, and OsAOS5 (Clade II). All Populus AOSs in Clade I contain a putative chloroplast transit peptide (likelihood>0.999), while the ones in Clade II do not. Within the AOS1 homologs, sequences from angiosperm and gymnosperms are grouped separately, with the exception for A. trichopoda. Amborella trichopoda is the most basal lineage in the clade of angiosperms, while its AOS gene groups with gymnosperm homologs.

3.2 Spatial Expression of OGY AOS1

AOS1 expression in petiole and phloem tissues was significantly higher compared to leaf and xylem (Fig. 2A). Expression difference between leaf and xylem, as well as between petiole and phloem, was not significant. This indicates that AOS1 is predominantly expressed in growing vascular tissues in Populus.

3.3 Motifs in Populus AOS Promoters

A total of 89 different motifs were identified in 508 locations of the OGY AOS1 promoter region. Most notably, there were six wounding signal sequences (TGACY), along with 28 pathogen/disease responsive motifs, 34 water stress-related motifs, 21 mesophyll-specific gene expression elements, 24 pollen-specific activation elements, and 92 hormone signaling elements. Among the hormone signaling elements, 28 have been reported for gibberellin induction, 27 for abscisic acid, 23 for cytokinin, 9 for salicylic acid, 3 for auxin and 1 for jasmonate. As a chloroplastic promoter, 92 motifs were found light responsive or regulated by phytochromes.

Similar to the OGY AOS1 promoter sequence, an average of 93 different motifs were identified in an average of 507 locations for the 11 P. trichocarpa and P. deltoides AOS homolog promoter regions. When motif abundance was compared between the two clades grouped in the phylogenetic tree (Fig. 1), 19 motifs were found to have significant differences (Table 1). Notably, promoters of AOS homologs in Clade I are more enriched with motifs that are hormone-, pathogen- and salt-responsive, as well as guard cell specific and anaerobically induced. In addition, we identified conserved promoter motifs associated with genes coding GAMOUS-like 15, DNA-binding with one finger (DoF) protein and beta-conglycinin. In contrast, higher abundance was found in Clade II motifs responding to water stress, heat shock, and CO$_2$. The OGY AOS1 promoter shares 82.2% and 71.2% identity, respectively, with the promoter region of Podel.02G143700.1 and Potri.002G130700.1.

3.4 Expression of β-glucuronidase (GUS) Driven by OGY AOS1 Promoter

When stained for GUS activity immediately after tissue harvest, blue staining was mainly localized in the cut sites, sepals and both ends of seed pods (Fig. 3). Roots were only lightly stained. No blue staining was found in the leaf except at the cut site. Physical wounding rapidly increased GUS activity (Fig. 4A). Dark blue staining was observed as early as 0.5 h after treatment and largely localized to wounded sites and vascular tissues. GUS staining was also conducted with unwounded leaves from the same plants at the same time points as the wounded leaves, and again we observed blue staining only at the...
excision sites, similar to the unwounded control at 0 h (Fig. 3A and Fig. 4A). Similar staining patterns were observed in all ten of the transgenic lines tested. Quantification by fluorometric essay, showed that GUS activity in leaf of 1 h treatment was significantly higher than the unwounded control ($P<0.01$) (Fig. 5).

4. DISCUSSION

This study shows that the promoter region of poplar AOS1 is activated in response to mechanical wounding. This result corroborates the findings in Frost et al. [16] and Lawrence et al. [31] that poplar AOS1 expression is responsive to wounding and herbivory. Furthermore, we demonstrate that the poplar AOS1 gene shows spatial variation in basal expression, with predominant expression in growing vascular tissues. The wounding response in AOS expression is rapid and being sustained for at least 24 hours in poplar [16] and for AOS promoter activation in Arabidopsis (Fig 4). Similarly, mechanical wounding has previously been reported to lead to systemic induction of AOS expression in tomato [2] and Arabidopsis [3,8]. In Oryza sativa, wound induction in OsAOS1 is transient while OsAOS2 is sustainable [13-14]. The Arabidopsis AOS is predominantly found in leaf and flower, with little expression in root, stem and silique [3]. Similar to Arabidopsis, wheat AOS has the highest expression in leaf [32]. In our study, poplar AOS was predominately expressed in stem. With such variety in responses, it is necessary to investigate the various AOS genes and promoter regions in different plant species rather than relying entirely on inference from model systems.

Table 1. Motifs identified in Populus AOS promoters* (~2 kb) that shows significant difference in abundance between Clade I and Clade II. P-value cutoff is 0.05. Conserved motifs were analyzed using a database of Plant Cis-acting Regulatory DNA Elements (PLACE) [26]

| Factor or Site Name | Signal Sequence | Annotation | Clade I | Clade II |
|---------------------|----------------|------------|---------|----------|
| EECCRCAH1           | (-) GANTTNC    | CO$_2$-responsive | 1.7     | 3.1      |
| -10PEHVPSBD         | (-) TATTCT     | Light-responsive | 4.0     | 2.8      |
| INRNTPSADB          | (-) YTCANTYY  | Light-responsive | 5.3     | 7.4      |
| CCAATBOX1           | (+) CCAAT      | Heat shock  | 3.0     | 4.4      |
| CARGCW8GAT          | (+)            | Binding site for AGAMOUS-like 15 | 4.7     | 2.7      |
| MYBCORE             | (-) CNGTTR     | Responsive to water stress | 0.3     | 4.7      |
| DOFCOREZM           | (+) AAAG       | Core site required for binding of Dof proteins | 46.7    | 26.3     |
| TATAPVTRNALEU       | (-) TTATATATA  | Frequently observed upstream of plant tRNA genes | 0.0     | 1.6      |
| CIACADIANLELHC      | (-) CAANNNATC  | Circadian; light | 1.7     | 2.1      |
| DRE1COREZMRAB17     | (-) ACCGAGA    | Drought-responsive element | 0.0     | 0.4      |
| ERELE4              | (-) AWTTCAAA   | Hormone response | 1.0     | 0.6      |
| ANAERO3CONSENSUS     | (+) TCATCAC    | Anaerobically induced | 1.0     | 0.2      |
| BOXCPAS1            | (+) CTCCCAC    | Light-induced | 1.3     | 0.1      |
| TAAAGSTKST1         | (+) TAAAG      | Guard cell-specific | 9.0     | 7.8      |
| MYB2CONSENSUSAT     | (+) YAACKG     | Dehydration-responsive abscisic acid signaling | 0.0     | 2.8      |
| SEF3MOTIFGM         | (+) AACCCA     | "SEF3 binding site"; consensus sequence found in the 5' upstream region of beta-conglycinin (7S globulin) gene | 4.7     | 0.9      |
| WUSATAg             | (+) TTAATGG    | Target sequence of WUS in the intron of AGAMOUS gene | 0.0     | 1.0      |
| GT1GMSCAM4          | (+) GAAAAA     | Pathogen- and salt-induced | 23.7    | 6.4      |
| PYRIMIDINEBOXHVEPB1 | (-) TTTTTC    | Gibberellin induction | 6.0     | 0.4      |

* Promoter sequences of Podel.14G039700.1, Podel.02G143700.1, Podel.04G152600.1, Podel.04G152500.1, Podel.09G112300.1, Potri.002G130700.1, Potri.014G038700.1, Potri.009G109700.1, Potri.004G149000.1, Potri.004G148900.1, Potri.004G148600.1, and poplar hybrid OGY AOS1
Fig. 1. Phylogenetic tree depicting the relationship of OGY AOS with homologs from other species. Sequences were aligned with ClustalW and the tree was constructed with MEGA X using the neighbor-joining method. Sequences were from either Phytozome or GenBank.

There are 89 different motifs in 508 locations in the ~2 kb OGY AOS1 promoter sequence we cloned. Consistent with the strong wound response, six wounding signal sequences (TGACY) are present. The 28 pathogen/disease responsive motifs we identified may play a role in the induction response by poplar leaf rust fungi [24]. It is noteworthy that there are 92 hormone signaling elements responsive to gibberellin, abscisic acid, cytokinin, salicylic acid, auxin, and jasmonate in the ~2 kb OGY AOS1 promoter sequence. While induction of AOS by hormone signals remains to be demonstrated in poplar, there are reports in peach (Ibuprofen and MeJA) [18], rice (jasmonate) [14], cacao (salicylic acid, ethylene, and MeJA) [20] and trifoliate orange (MeJA and abscisic acid) [23]. The conserved motifs are largely similar among Populus AOS promoters, while there is significant difference in abundance of the 19 sequences between homologs separated into the two clades phylogenetically. This suggests that the differences in motifs may explain the discrepancies in gene expression and function among these homologs. Considering the critical roles that Dof proteins play in plant growth and development, the high abundance of the Dof-related motif (CNGTTR) in clade I promoters can be an indication that clade I AOS homologs (AOS1s) may be involved in plant development. Results from the motif analysis also suggest that clade I AOS genes are more likely responsive to hormones, particularly gibberellins (6.0 in clade I vs 0.4 in clade II) and pathogen/salt (23.7 in clade I vs. 6.4 in clade II), while clade II genes are more inducible by water stress (0.3 in clade I vs. 7.9 in clade II).
AOS1 expression in leaf, petiole, phloem and xylem of hybrid poplar OGY analyzed by RT-qPCR. Relative AOS1 expression was normalized to 18S. Bars represent means +/- standard error of the mean (SEM) of three biological replicates. Different letters above the bars reflect statistical difference at $\alpha=0.05$

AOS is found as single gene in Arabidopsis. Mining the genome of two Populus species, P. trichocarpa and P. deltoides, resulted in identification of six and five AOS homologs, respectively, which group into two separate sister clades. Two gene models from P. trichocarpa Potri.002G130700 and Potri.014G038700.1 and P. deltoides (Podel.02G143700.1 and Podel.14G039700.1) are in Clade I along with OGY AOS1, AtAOS, and OsAOS1, therefore, they are named AOS1. The other poplar AOS homologs are grouped in Clade II. The phylogenetic clustering suggests that there are two types of AOS1 in the Populus genome. This is consistent with the analysis by [33]. Whether the duplicated poplar AOS1 genes have redundant or complementary activities remains to be investigated. Proteins in Clade I contain a chloroplast transit signal sequence. In tomato it was demonstrated that this signal peptide targets AOS to the chloroplast inner envelope membrane [34]. The remaining Populus AOSs are in Clade II, grouped with OsAOS2-4 and two barley homologs. Functionally different from OsAOS1, the OsAOS2 and OsAOS3 genes are not responsive to light treatment [13]. Additional comprehensive studies are warranted to understand the similarities and differences in the expression patterns of poplar AOSs.

In recent years, there is evidence that jasmonate promotes auxin-induced adventitious rooting [35-36]. All promoters of the Clade I poplar AOS genes contain one root hair-specific cis-elements (RHERPATEXP7, KCACGW). In Clade II, promoters of Potri.009G109700.1, Potri.009G112300.1, Potri.004G148900.1 and Potri.04G152500.1 have 1, 3, 4, and 5 such cis-elements, respectively. The epidermal (L1) layer-specific motif, L1BOXATPDF1 (TAAATGYA) exists in three of the poplar AOS promoters. It is speculated that some of the poplar AOS genes may play a role in ease of formation of adventitious roots by poplar cuttings. Additionally, because of its rapid and localized activation at wounded sites, the OGY AOS1 promoter could be utilized to drive rooting promoting gene expression in species such as chestnuts and camellias that are recalcitrant to rooting in cuttings.
Fig. 3. GUS staining of OGY AOS1 promoter::GUS transgenic Arabidopsis. Tissues were submerged in X-Gluc solution immediately after harvest and stained overnight. A: leaf; B and C: floral stem; D: root; E and F: flower and seed pod. The scale bars represent one centimeter.

Fig. 4. GUS staining of OGY AOS1 promoter::GUS transgenic Arabidopsis under physical wounding. A portion of a leaf was crushed with a pair of serrated forceps, Whole leaves were submerged in X-Gluc solution after 0.5 h, 1 h, 2 h, 6 h and 24 h immediately after harvest and stained overnight. A: without 70 % ethanol wash; B: with 70 % ethanol wash (1 h after wounding).
Fig. 5. Quantification of GUS activity under the control of poplar OGY AOS1 promoter using a fluorometric essay. Leaves of transgenic Arabidopsis plants carrying OGY AOS1 promoter::GUS transgenic were wounded for one hour and harvested for the GUS assay. The error bars represent standard deviation. Significant differences existed between wounded and unwounded samples within each transgenic line (T1 to T5) (P<0.01), represented by two different letters.

5. CONCLUSION

In conclusion, as the first committed enzyme in the lipoxygenase pathway that leads to the biosynthesis of jasmonic acid and its derivatives, much progress has been made in understanding the roles of AOS in plant defense and development. Our study provides insight on the diversity of responsive elements in the poplar AOS promoters, and that the differences between homologs in separate clades suggest differences in roles that AOS genes may play in plant development and responses to biotic and abiotic stress. Our results also indicate that diversity of motifs in AOS gene promoters provide a wide range of opportunities for targeting gene expression to various environmental conditions and developmental stages in plants.

SUPPLEMENTARY MATERIALS

Supplimentary materials available in this link: https://www.journalbji.com/index.php/BJI/libraryFiles/downloadPublic/12.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Farmer EE, Goossens A. Jasmonates: what allene oxide synthase does for plants. J. Exp. Bot. 2019;70:3373-3378. Available: https://doi.org/10.1093/jxb/erz254

2. Howe GA, Lee GI, Itoh A, Li L, DeRocher AE. Cytochrome P450-dependent metabolism of oxylipins in tomato. Cloning and expression of allene oxide synthase and fatty acid hydroperoxide lyase. Plant Physiol. 2000;123:711-724. Available: https://doi.org/10.1104/pp.123.2.711
3. Laudert D, Pfannschmidt U, Lottspeerch F, Hollander-Czytko H, Weiler EW. Cloning, molecular and functional characterization of Arabidopsis thaliana allene oxide synthase (CYP 74), the first enzyme of the octadecanoid pathway to jasmonates. Plant Mol. Biol. 1996;31:323-335. Available:https://doi.org/10.1007/BF00021793

4. Maucher H, Hause B, Feussner I, Ziegler J, Wasternack C. Allene oxide synthases of barley (Hordeum vulgare cv. salome): tissue specific regulation in seedling development. Plant J. 2000;21:199-213. Available:https://doi.org/10.1046/j.1365-313x.2000.00669.x

5. Peng Q, Zhou Y, Liao Y, Zeng L, Xu X, Jia Y, Dong F, Li J, Tang J, Yang Z. Functional characterization of an allene oxide synthase involved in biosynthesis of jasmonic acid and its influence on metabolite profiles and ethylene formation in tea (Camellia sinensis) flowers. Int. J. Mol. Sci. 2018;19. Available:https://doi.org/10.3390/ijms1908440

6. Placido DF, Dierig DA, Cruz VMV, Ponciano G, Dong C, Dong N, Huynh T, Williams T, Cahoon RE, Wall GW, Wood DF, McMahan C. Downregulation of an allene oxide synthase gene improves photosynthetic rate and alters phytohormone homeostasis in field-grown guayule. Ind. Crops Prod. 2020;153:112341. Available:https://doi.org/10.1016/j.indcrop.2020.112341

7. Wilmowicz E, Kučko A, Frankowski K, Zbrocka-Nowakowska B, Panek K, Kopećewicz J. Wounding stimulates allene oxide synthase gene and increases the level of jasmonic acid in Ipomoea nil cotyledons. Acta Soc. Bot Pol. 2016;85. Available:https://doi.org/10.5586/asbp.3491

8. Kubigsteltig I, Laudert D, Weiler EW. Structure and regulation of the Arabidopsis thaliana allene oxide synthase gene. Planta. 1999;208:463-471. Available:https://doi.org/10.1007/s004250050583

9. Tamaoki M, Freeman JL, Pilon-Smets EA. Cooperative ethylene and jasmonic acid signaling regulates selenite resistance in Arabidopsis. Plant Physiol. 2008;146:1219-1230. Available:https://doi.org/10.1104/pp.107.110742

10. Naor N, Gurung FB, Ozalvo R, Bucki P, Sanadhya P, Miyara SB. Tight regulation of allene oxide synthase (AOS) and allene oxide cyclase-3 (AOC3) promote Arabidopsis susceptibility to the root-knot nematode Meloidogyne javanica. Eur. J. Plant Pathol. 2018;150:149-165. Available:https://doi.org/10.1007/s10658-017-1261-2

11. Song WC, Brash AR. Purification of an allene oxide synthase and identification of the enzyme as a cytochrome P-450. Science. 1991;253:781-784. Available:https://doi.org/10.1126/science.1876834

12. Itoh A, Schilimiller AL, McCaig BC, Howe GA. Identification of a jasmonate-regulated allene oxide synthase that metabolizes 9-hydroperoxides of linoleic and linolenic acids. J. Biol. Chem. 2002;277:46051-46058. Available:https://doi.org/10.1074/jbc.M207234200

13. Haga K, Iino M. Phytochrome-mediated transcriptional up-regulation of allene oxide synthase in rice seedlings. Plant Cell Physiol. 2004;45:119-128. Available:https://doi.org/10.1093/pcp/pch025

14. Agrawal GK, Rakwal R, Jwa N-S, Han K-S, Agrawal VP. Molecular cloning and mRNA expression analysis of the first rice jasmonate biosynthetic pathway gene allene oxide synthase. Plant Physiol. Bioch. 2002;40:771-782. Available:https://doi.org/10.1016/s0981-9428(02)01429-8

15. Gnanaprakash HP, Jogaiah S, Sreedhara AP, Prashanth NG, Kini RK, Shetty SH. Association between accumulation of allene oxide synthase activity and development of resistance against downy mildew disease of pearl millet. Mol. Biol. Rep. 2013;40:6821-6829. Available:https://doi.org/10.1007/s11033-013-2799-5

16. Frost CJ, Mescher MC, Dervinis C, Davis JM, Carlson JE, De Moraes CM. Priming defense genes and metabolites in hybrid poplar by the green leaf volatile cis-3-hexenyl acetate. New Phytol. 2008;180:722-734. Available:https://doi.org/10.1111/j.1469-8137.2008.02599.x
17. Siqueira-Junior CL, Jardim BC, Urmenyi TP, Vicente AC, Hansen E, Otsuki K, da Cunha M, Madureira HC, de Carvalho DR, Jacinto T. Wound response in passion fruit (Passiflora f. edulis flavicarpa) plants: gene characterization of a novel chloroplast-targeted allene oxide synthase up-regulated by mechanical injury and methyl jasmonate. Plant Cell Rep. 2008;27:387-397. https://doi.org/10.1007/s00299-007-0451-3
18. Torrigiani P, Fregola F, Ziosi V, Ruiz KB, Kondo S, Costa G. Differential expression of allene oxide synthase (AOS), and jasmonate relationship with ethylene biosynthesis in seed and mesocarp of developing peach fruit. Postharvest Biol. Technol. 2012;63:67-73. https://doi.org/10.1016/j.postharvbio.2011.08.008
19. Dumin W, Rostas M, Winefield C. Identification and functional characterisation of an allene oxide synthase from grapevine (Vitis vinifera L. Sauvignon blanc). Mol. Biol. Rep. 2018;45:263-277. https://doi.org/10.1007/s11033-018-4159-y
20. Litholdo CG, Jr., Leal GA, Jr., Albuquerque S, Machado H, Costa RL, Duque P, Malho R. Expression of castanea crenata allene oxide synthase in arabidopsis improves the defense to phytophthora cinnamononi. Front. Plant Sci. 2021;12:628697. https://doi.org/10.3389/fpls.2021.628697
21. Wang H, Gao Z, Liu H, Wu J, Yu D. Variation in GmAOS1 promoter is associated with soybean defense against insect attack. Euphytica. 2014;196:365-374. https://doi.org/10.1007/s10681-013-1038-4
22. Xiong J, Liu L, Ma X, Li F, Tang C, Li Z, Lu B, Zhou T, Lian X, Chang Y, Tang M, Xie S, Lu X. Characterization of PtAOS1 promoter and three novel Interacting proteins responding to drought in Poncirus trifoliata. Int. J. Mol. Sci. 2020;21. Available:https://doi.org/10.3390/ijms21134705
23. Azraiez A, Boyle B, Levee V, Seguin A. Transcriptome profiling in hybrid poplar following interactions with melampsora rust fungi. Mol. Plant Microbe Interact. 2009;22:190-200. Available:https://doi.org/10.1094/MPMI-22-2-0190
24. Corpet F. Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res. 1988;16:10881-10890.
25. Chang S, Puryear J, Cairney J. A simple and efficient method for isolating RNA from pine trees. Plant Mol. Biol. 1993;11:113-116.
26. Higo K, Ugawa Y, Iwamoto M, Korenaga T. Plant cis-acting regulatory DNA elements (PLACE) database. Nucleic Acids Res. 1999;27:297–300. Available:https://doi.org/10.1093/nar/27.1.297
27. Desfeux C, Clough SJ, Bent AF. Female reproductive tissues are the primary target of agrobacterium-mediated transformation by the arabidopsis floral-dip method. Plant Physiol. 2000;123:895-904. Available:https://doi.org/10.1104/pp.123.3.895
28. Xu Y, Thammannagowda S, Thomas TP, Azadi P, Schlarbaum SE, Liang H. LtuCAD1 is a cinamyl alcohol dehydrogenase ortholog involved in lignin biosynthesis in Liriodendron tulipifera L., a basal angiosperm timber species. Plant Mol. Biol. Rep. 2013;31:1089-1099.
29. Jefferson RA, Kavanagh TA, Bevan MW. GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 1987;6:3901-3907.Chang S, Puryear J, Cairney J. A simple and efficient method for isolating RNA from pine trees. Plant Mol. Biol. 1993;11:113-116.
30. Lawrence SD, Dervinis C, Novak N, Davis JM. Wound and insect herbivory responsive genes in poplar. Biotechnol. Lett. 2006;28:1493-1501. Available:https://doi.org/10.1007/s10529-006-9119-2
31. Liu H-H, Wang Y-G, Wang S-P, Li H-J, Xin Q-G. Improved zinc tolerance of tobacco by transgenic expression of an allene oxide synthase gene from hexaploid wheat. Acta Physiol. Plant. 2014;36:2433-2440.
32. Herr JR. Soil microbial diversity and the influence on stress response in hybrid poplar. A Doctoral Dissertation at the Pennsylvania State University; 2013.
33. Froehlich JE, Itoh A, Howe GA. Tomato allene oxide synthase and fatty acid
hydroperoxide lyase, two cytochrome P450s involved in oxylipin metabolism, are targeted to different membranes of chloroplast envelope. Plant Physiol. 2001;125:306-317.

35. Fattorini L, Hause B, Gutierrez L, Veloccia A, Della Rovere F, Piacentini D, Falasca G, Altamura MM. Jasmonate promotes auxin-induced adventitious rooting in dark-grown Arabidopsis thaliana seedlings and stem thin cell layers by a cross-talk with ethylene signalling and a modulation of xylogenesis. BMC Plant Bio. 2018;18:1-18.

36. Xu P, Zhao P-X, Cai X-T, Mao J-L, Miao Z-Q, Xiang C-B. Integration of jasmonic acid and ethylene into auxin signaling in root development. Front. Plant Sci. 2020;11:271.