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

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Authors' contributions

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

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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 ß-glucuronidase (*GUS*) gene in *Arabidopsis*. AOS is an

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essential 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.

Keywords: Gene expression; herbivory; non-systemic expression; populous.

Key Message

- *Populus* AOSs separate phylogenetically into two clades, which show significant abundance differences in 19 promoter motifs.
- *AOS*1 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, both *AtAOS* mRNA and protein levels increased in wounded and systemic leaves [3], mirroring the ß-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* transcription 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'AAACATGGCTTCCTCTTCC3' and 5'CTCGGAAAGCATTGGGTAA3') 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 plastrochon 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′AATTGTTGGTCTTCAACGAA3′, reverse: 5′AAADDDCAGGGACGTAGTCAA3′) 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 ß-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'GAAGCCATGTTTGGGATTTT3' and 5'GGAAAACAAATGGGGAAA3') and the *GUS* gene (5'TGTGTCTATGATGATGATG3' 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. chichocarpa*, *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□V□NKQCAG of the heme binding domain and the highly conserved motif □G□KIL of the CYP74A enzymes were found in *OGY* AOS1.

Six *AOS* homologs were identified in the *P. trichocarpa* genome when using *AtAOS* 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 *AtAOS* 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. The identity ranged from 33.0% to 17.8% for the other *P. trichocarpa* and *P. deltoides AOS* sequences. All conserved motifs identified in poplar *AOS* promoters are listed in Supplemental Table 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	(-) CWWWWWWWWG	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	(-) TTTATATA	Frequently observed upstream of plant tRNA genes	0.0	1.6
CIACADIANLELHC	(-) CAANNNNATC	Circadian; light	1.7	2.1
DRE1COREZMRAB17	(-) ACCGAGA	Drought-responsive element	0.0	0.4
ERELEE4	(-) AWTTCAAA	Hormone response	1.0	0.6
ANAERO3CONSENSUS	(+) TCATCAC	Anaerobically induced	1.0	0.2
BOXCPSAS1	(+) 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	(-) TTTTTTCC	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*

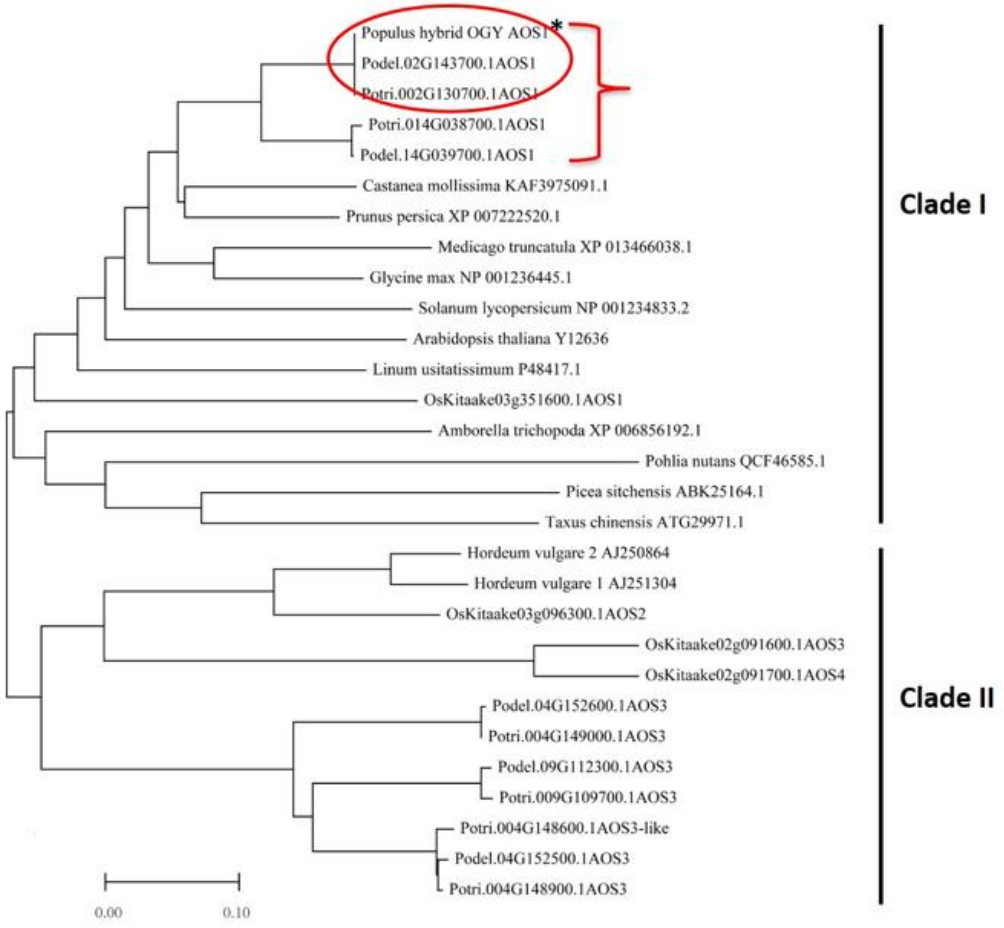

Fig. 1. Phylogenetic tree depicting the relationship of *OGY AOS* with homologs from other species. Sequences were aligned with ClustalW and the tree was constructed with MEGA X using the neighbor-joining method. Sequences were from either Phytozome or GenBank

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).

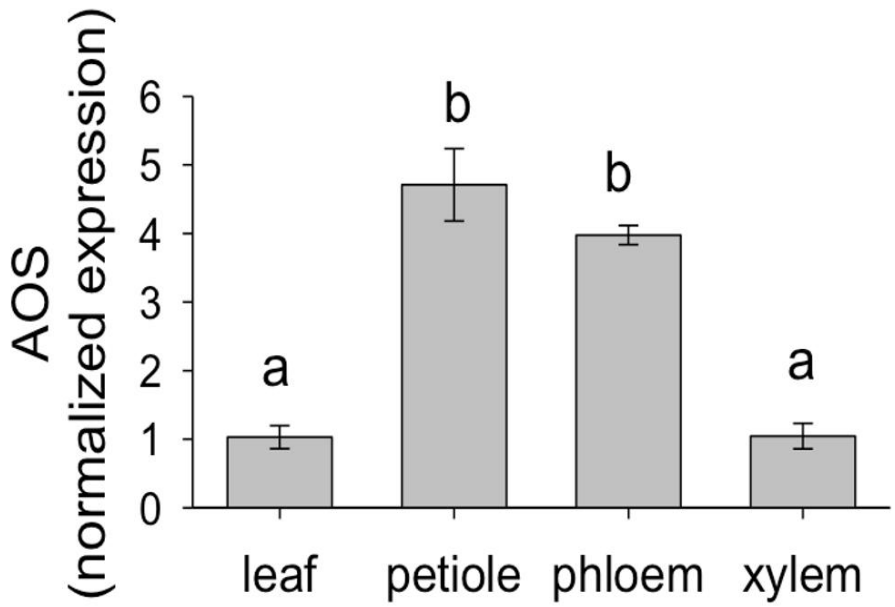

Fig. 2. *AOS1* expression in leaf, petiole, phloem and xylem of hybrid poplar *OGY* analyzed by RT-qPCR. Relative *AOS1* expression was normalized to 18S. Bars represent means +/- standard error of the mean (SEM) of three biological replicates. Different letters above the bars reflect statistical difference at α=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, *At*AOS, and *Os*AOS1, 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 *Os*AOS2-4 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 (RHERPATEXPA7, KCACGW). In Clade II, promoters of Potri.009G109700.1, Podel.09G112300.1, Potri.004G148900.1 and Podel.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.

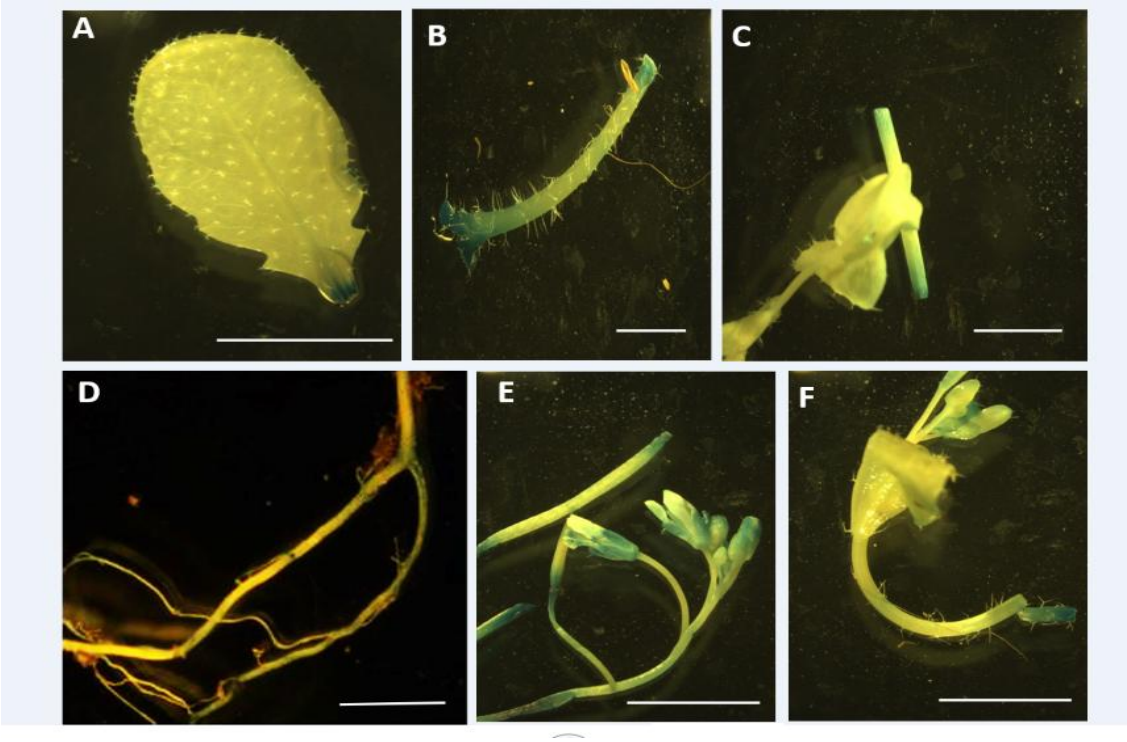

Fig. 3. GUS staining of *OGY AOS1* promoter*::GUS* transgenic *Arabidopsis*. Tissues were submerged in X-Gluc solution immediately after harvest and stained overnight. A: leaf; B and C: floral stem; D: root; E and F: flower and seed pod. The scale bars represent one centimeter

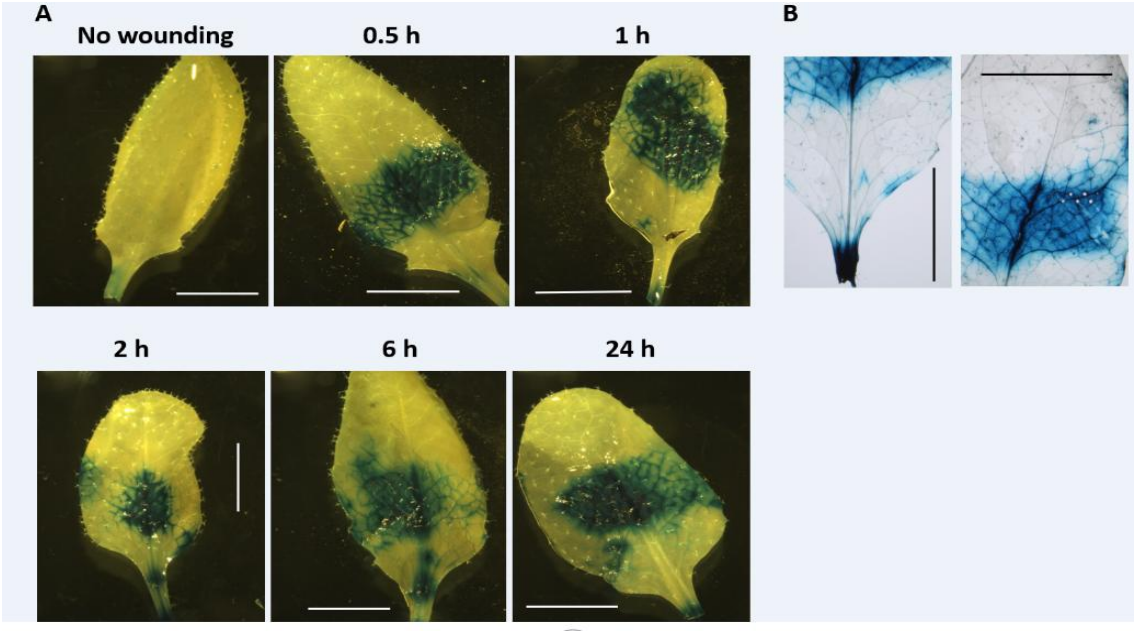

Fig. 4. GUS staining of *OGY AOS1* promoter*::GUS* transgenic *Arabidopsis* under physical wounding. A portion of a leaf was crushed with a pair of serrated forceps, Whole leaves were submerged in X-Gluc solution after 0.5 h, 1 h, 2 h, 6 h and 24 h immediately after harvest and stained overnight. A: without 70 % ethanol wash; B: with 70 % ethanol wash (1 h after wounding)

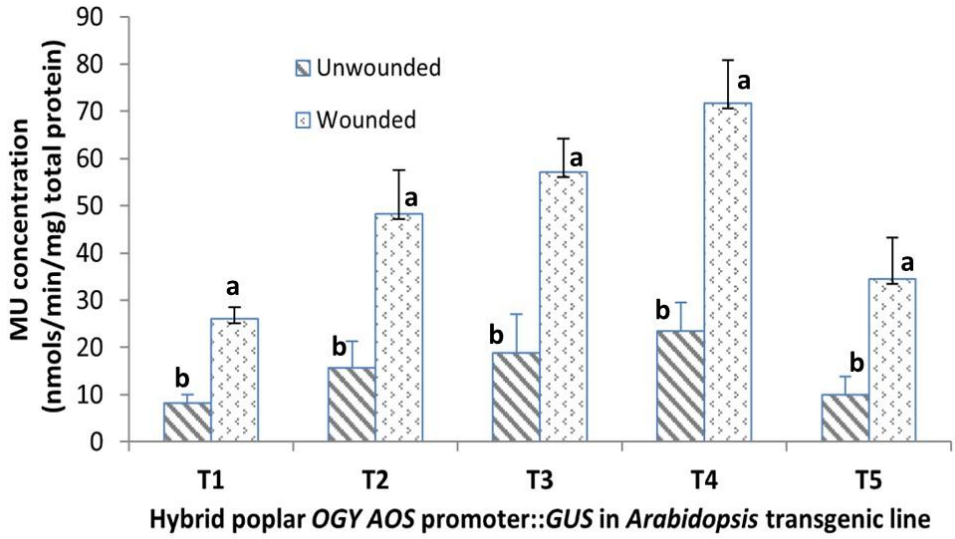

Fig. 5. Quantification of GUS activity under the control of poplar *OGY AOS1* promoter using a fluorometric essay. Leaves of transgenic *Arabidopsis* plants carrying *OGY AOS1* promoter::*GUS* transgenic were wounded for one hour and harvested for the GUS assay. The error bars represent standard deviation. 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 in 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.

SUPPLIMENTARY 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.

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