Cloning and Tissue Expression Analysis of the AOX Gene Family in *Liriodendron chinense* (Postprint)

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Abstract

Alternative oxidase (AOX) is the terminal oxidase of the plant respiratory electron transport chain and is widely present in higher plants. Studies have shown that AOX plays an important role in plant growth, development, and adaptation. Liriodendron chinense is an important precious timber and ornamental landscape tree species. Research on stress resistance genes is of great significance for improving the adaptability of Liriodendron chinense. This study used Liriodendron chinense as the research object and cloned three AOX genes using a combined RT-PCR and RACE approach. Their ORF lengths were 858 bp, 1,032 bp, and 1,044 bp, respectively, encoding 285 aa, 343 aa, and 347 aa amino acids, which were named LcAOX1a, LcAOX1b, and LcAOX2, respectively. Protein homology analysis revealed that the AOX family protein sequences in Liriodendron chinense are highly conserved, particularly at the C-terminus, and all contain the "EXXH" and "EEE-Y" iron ion-binding conserved domains. Subcellular localization analysis showed that the LcAOX1a protein is localized to locations other than mitochondria and chloroplasts, the LcAOX1b protein is localized in both chloroplasts and mitochondria, and the LcAOX2 protein is localized in the mitochondrial matrix. Using RT-qPCR, the expression patterns of AOX genes were investigated in eight different tissues of Liriodendron chinense: stem, leaf, leaf bud, flower bud, calyx, petal, stamen, and pistil. The analysis revealed that AOX genes were expressed significantly higher in floral organs than in vegetative organs. LcAOX1a and LcAOX1b genes showed the highest expression in stamens, with LcAOX1a being specifically expressed in stamens at levels far exceeding those in other tissues. In contrast, LcAOX2 exhibited the highest expression in petals. This study cloned three AOX genes and conducted relevant analyses, laying a foundation for further investigation of their biological functions.

Full Text

Molecular Isolation and Expression Analysis of Alternative Oxidase Genes in *Liriodendron chinense*

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Abstract

Alternative oxidase (AOX) is a terminal oxidase in the plant respiratory electron transport chain that is widely distributed in higher plants. Research has demonstrated that AOX plays important roles in plant growth, development, and environmental adaptation. *Liriodendron chinense* is a valuable timber and ornamental tree species, and investigating stress-resistance genes is crucial for improving its adaptability. In this study, we cloned three AOX genes from *L. chinense* using a combination of RT-PCR and RACE techniques. Their open reading frames (ORFs) were 858 bp, 1,032 bp, and 1,044 bp in length, encoding 285, 343, and 347 amino acids, respectively, which were designated as *LcAOX1a*, *LcAOX1b*, and *LcAOX2*. Protein homology analysis revealed that the AOX family proteins in *L. chinense* were highly conserved, particularly at the C-terminus, and all contained the "EXXH" and "EEE-Y" iron-binding conserved domains. Subcellular localization analysis showed that LcAOX1a protein was localized outside mitochondria and chloroplasts, LcAOX1b protein was localized in both chloroplasts and mitochondria, and LcAOX2 protein was localized in the mitochondrial matrix. Using RT-qPCR, we examined the expression patterns of AOX genes in eight different tissues: stem, leaf, leaf bud, flower bud, calyx, petal, stamen, and pistil. The results indicated that AOX genes were expressed at significantly higher levels in floral organs than in vegetative organs. Both *LcAOX1a* and *LcAOX1b* showed the highest expression in stamens, with *LcAOX1a* being specifically and abundantly expressed in stamens at levels far exceeding those in other tissues. In contrast, *LcAOX2* exhibited the highest expression in petals. This study cloned three AOX genes and conducted relevant analyses, laying a foundation for further investigation of their biological functions.

Keywords: *Liriodendron chinense*, alternative oxidase, gene cloning, bioinformatics analysis, tissue expression

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Higher plant mitochondria possess two important electron transport pathways: the cytochrome pathway and the alternative pathway (also known as the cyanide-resistant respiratory pathway). Under normal conditions, plants primarily use the cytochrome pathway, where electrons are transferred through a series of complexes and ultimately to oxygen to form water, with the generated chemical energy coupled to ATP synthesis via F0F1-ATPase. This pathway

contains three phosphorylation sites and is sensitive to cyanide. Under stress conditions, the cytochrome pathway is inhibited while the alternative pathway is enhanced. The alternative pathway branches from the ubiquinone pool and uses alternative oxidase (AOX) as the terminal oxidase, transferring electrons directly from reduced ubiquinone to molecular oxygen to form water, with energy dissipated as heat (Vanlerberghe et al., 1997). Previous studies have shown that AOX gene expression and protein abundance increase significantly in response to adverse environmental conditions, helping cells cope with stress-induced damage.

Bonner & Rich (1986) first isolated AOX from mitochondria of *Arum maculatum* (Araceae) and identified its association with thermogenesis during flowering. Zalutskaya et al. (2015) reported that alternative respiration was significantly enhanced and *AOX1* expression was upregulated in *Chlamydomonas reinhardtii* after heat treatment. Additionally, enhanced alternative respiration has been observed in Arabidopsis under cold and drought stress, in *Medicago truncatula* under salt stress, and transgenic plants overexpressing AOX showed significantly improved stress tolerance (Chinnusamy et al., 2007; Campos et al., 2016; Jian et al., 2016; Keunen et al., 2016).

Liriodendron chinense (Magnoliaceae) is a tertiary relict species and one of the most primitive groups of angiosperms. Endemic to China, this species has poor natural regeneration, low seed propagation fitness, small natural populations, and limited individual numbers. It is sporadically distributed in southwestern and central-eastern China and northern Vietnam, with severely restricted gene flow between populations, placing it in an endangered state. It has been listed as a national second-class rare and endangered protected plant (Hao et al., 1995). As a transitional species between gymnosperms and angiosperms, *L. chinense* holds important value for studying the origin, distribution, and phylogeny of flowering plants (Li, 2013). Moreover, as an excellent ornamental and high-quality timber tree, it possesses significant economic value. Broad adaptability is a prerequisite for species utilization and promotion. Therefore, from the perspective of forest genetic improvement, mining and studying stress-related genes to enhance the adaptability of *L. chinense* is of great significance for resistance breeding. Based on this, the present study used *L. chinense* as experimental material, employing RACE technology to screen and clone AOX family genes, conducting bioinformatics analysis and tissue expression characterization to lay a foundation for future research on their stress resistance functions.

Materials and Methods

Plant Materials

All tissue materials including leaves, stems, leaf buds, flower buds, calyx, petals, stamens, and pistils were collected from *L. chinense* (provenance: Songyang, Zhejiang) growing in the *Liriodendron* provenance trial forest at the Xiashu Experimental Forest Farm of Nanjing Forestry University. Samples were rapidly

frozen in liquid nitrogen immediately after collection and stored at -80°C until use. *Escherichia coli* competent cells (Trans1-T1 Phage Resistant Chemically Competent Cell), pEASY-T1 Cloning Kit, and pEASY-Blunt Cloning Kit were purchased from TransGen Biotech (Beijing).

1.2.1 Mining of AOX Family Gene EST Sequences

The *Liriodendron tulipifera* transcriptome database (http://ancangio.uga.edu/content/liriodendron-tulipifera) was searched to identify all AOX gene EST fragments, which were then exported. These EST fragments were BLAST-aligned against our laboratory' s *L. chinense* transcriptome data to screen for AOX homologous gene EST fragments.

1.2.2 RNA Extraction and First-Strand cDNA Synthesis

Total RNA was extracted from mixed samples of *L. chinense* leaves, petals, stamens, and pistils using the RNAprep pure Plant Kit (DP432) from TIANGEN. First-strand cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit from Thermo Fisher Scientific. For 3' RACE, the 3' -Full RACE Core Set with PrimeScript™ RTase (TaKaRa) was used, while the SMARTer® RACE 5' /3' Kit (TaKaRa) was employed for 5' RACE. RNA integrity was verified before reverse transcription to ensure no degradation.

1.2.3 Full-Length cDNA Sequence Acquisition

Based on the obtained EST sequences, specific primers for intermediate fragments were designed using Oligo 7 software (Table 1). Using the first-strand cDNA as template, PCR amplification was performed with LA Taq (TaKaRa). The amplified products were detected by 1.5% agarose gel electrophoresis, and target fragments were excised, purified, ligated into the pEASY-T1 vector, and transformed into Trans1-T1 competent cells. Positive clones were sequenced by GenScript (Nanjing) to obtain intermediate fragment sequences. Based on these sequences, 3' and 5' RACE primers were designed (Table 1) using a nested approach. Outer and inner PCR reactions were performed to rapidly amplify the 3' and 5' ends of the target genes. The intermediate fragments, 3' RACE, and 5' RACE sequences were electronically assembled using BioXM software. The assembled full-length sequences were compared against the NCBI database, and ORF sequences were predicted using the ORF Finder online tool. Full-length primers were designed based on the predicted ORF sequences (Table 1) to amplify the ORF sequences.

1.2.4 Bioinformatics Analysis

Bioinformatics software was used to analyze the obtained AOX genes, including open reading frames, encoded amino acid sequences, protein domains, and secondary structures. ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/) was used for ORF and amino acid sequence prediction. Protein amino

acid composition, molecular weight, and theoretical isoelectric point were analyzed using ExPASy ProtParam (http://web.expasy.org/protparam/). Protein domain analysis was performed using Pfam (http://pfam.xfam.org/). Transmembrane regions were analyzed using the online tool ExPASy TMpred (http://www.ch.embnet.org/software/TMPRED_form.html). Signal peptide analysis was conducted using SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). Protein secondary structure was predicted using SOPMA. Subcellular localization was predicted using TargetP 1.1 Server (http://www.cbs.dtu.dk/services/TargetP/) and Wolfpsort (https://wolfpsort.hgc.jp/). Published AOX homologous sequences were downloaded from NCBI for analysis, and phylogenetic trees were constructed using MEGA5.1.

1.2.5 Tissue Expression Analysis

Total RNA was extracted from eight different tissues of *L. chinense*: leaf, stem, leaf bud, flower bud, calyx, petal, stamen, and pistil. RNA samples were diluted to a uniform concentration (250 ng · μL ^{1}) and reverse-transcribed into cDNA. Based on the cloned *AOX1a*, *AOX1b*, and *AOX2* gene sequences, qPCR primers were designed using Oligo 7 software (Table 1). *Actin97* was used as the reference gene for real-time quantitative PCR to detect target gene expression in the eight tissues, with three replicates per sample. The reaction system and protocol followed the instructions for SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (TaKaRa).

Results

2.1 Cloning and Nomenclature of Full-Length AOX Family Gene cDNAs from *L. chinense*

Sixteen AOX gene-related EST fragments were identified from the *L. tulipifera* transcriptome database. After BLAST alignment and deduplication with our laboratory's *L. chinense* transcriptome data (Yang, 2013), three AOX gene EST fragments were obtained: lcomp84206_c0, lcomp98789, and lcomp99880_c0. Specific primers for intermediate fragments were designed based on these EST sequences. PCR amplification, fragment recovery, and sequencing verified the AOX gene sequences in the transcriptome (Figure 1 [Figure 1: see original paper]: A). RACE primers were then designed based on the intermediate fragment sequencing results, and nested amplification was used to obtain 3' RACE (Figure 1: C) and 5' RACE (Figure 1: D) products. The intermediate fragments, 3' RACE, and 5' RACE sequences were electronically assembled to obtain full-length sequences, and primers flanking the ORF were designed for validation (Figure 1: B), confirming the assembly accuracy. The three cloned AOX genes were predicted and compared against the NCBI database using BLAST Protein. Based on the results, the genes were designated as *LcAOX1a* (MN187966), *LcAOX1b* (MN187968), and *LcAOX2* (MN187967).

2.2 Physicochemical Properties of Encoded Proteins

All three AOX proteins contained conserved AOX domains. *LcAOX1a* and *LcAOX1b* belonged to the AOX1 subfamily, while *LcAOX2* was a member of the AOX2 subfamily. The *LcAOX1a* gene was 1,305 bp in length with an ORF of 858 bp encoding 285 amino acids, a theoretical isoelectric point of 7.23, a grand average of hydropathicity of -0.264, an aliphatic index of 80.11, and an instability coefficient of 36.17. The *LcAOX1b* gene was 1,405 bp in length with an ORF of 1,032 bp encoding 343 amino acids, a theoretical isoelectric point of 8.37, a grand average of hydropathicity of -0.181, an aliphatic index of 85.04, and an instability coefficient of 39.71. The *LcAOX2* gene was 1,376 bp in length with an ORF of 1,044 bp encoding 347 amino acids, a theoretical isoelectric point of 9.02, a grand average of hydropathicity of -0.251, an aliphatic index of 89.94, and an instability coefficient of 48.17. Analysis of transmembrane regions using the online tool ExPASy TMpred revealed that AOX proteins are typical transmembrane proteins, with regions of high hydrophobicity variation corresponding to their transmembrane domains.

2.3 Homology Analysis of AOX Family Proteins

The *L. chinense* AOX family proteins LcAOX1a, LcAOX1b, and LcAOX2 were compared against sequences in the NCBI database. The results indicated that AOX family protein sequences were highly conserved, particularly at the C-terminus (Figure 2 [Figure 2: see original paper]). AOX proteins from different species all contained the characteristic "EXXH" and "EEE-Y" iron-binding conserved domains. Six conserved histidine residues likely participate in iron ion binding, while one conserved serine residue is involved in disulfide bond formation. Therefore, we hypothesize that the cloned *LcAOX1a*, *LcAOX1b*, and *LcAOX2* genes, as members of the diiron carboxylate superfamily, possess iron-binding activity.

2.4 Phylogenetic Analysis of AOX Family Proteins

Nineteen AOX family homologous proteins were retrieved from the NCBI protein database, including sequences from *Arabidopsis thaliana* (NP_188876.1, NP_188875.1, NP_189399.1, NP_201226.2), rice (*Oryza sativa*, *Oryza glaberrima*) (BAA28773.1, BAB71945.1), carrot (*Daucus carota* var. *sativa*) (ALI57378.1, ABZ81229.2, ABZ81230.2), grape (*Vitis vinifera*) (ACI28876.1, NP_001268001.1), tomato (*Lycopersicon esculentum*) (NP_001234117.2, NP_001309890.1), soybean (*Glycine max*) (KHN42869.1, KHN39226.1), and watermelon (*Citrullus lanatus*) (ADD84880.1). The three *L. chinense* AOX proteins and the retrieved sequences were aligned using MEGA5.0, and a phylogenetic tree was constructed using the maximum likelihood method (Figure 3 [Figure 3: see original paper]). The AOX proteins clearly divided into two major clades: AOX1 and AOX2. Phylogenetically, LcAOX1a and LcAOX1b formed a small branch most closely related to carrot DcAOX1, while LcAOX2 was closely related to carrot DcAOX2a and other proteins.

These results suggest that *Liriodendron* is more closely related to carrot in evolutionary terms and confirm the classification of AOX family members into AOX1 and AOX2 subfamilies.

2.5 Subcellular Localization Prediction

Subcellular localization was predicted using TargetP online software (Table 2). The results indicated that LcAOX1a protein might be distributed in locations other than chloroplasts, mitochondria, and the secretory pathway. LcAOX2 protein was predicted to localize to mitochondria with a reliability class of 2 (high reliability). Although LcAOX1b showed the highest score for mitochondrial localization, its specific localization was unclear. To improve prediction accuracy, Wolfpsort online software was used for additional analysis (Table 3). The results showed that LcAOX1a was most likely localized in the cytoplasm, LcAOX2 in mitochondria, and LcAOX1b in both mitochondria and chloroplasts, though experimental validation is required for definitive localization.

2.6 Tissue Expression Pattern Analysis

Total RNA was extracted from eight tissues of *L. chinense* (leaf, stem, leaf bud, flower bud, calyx, petal, stamen, and pistil) and reverse-transcribed into cDNA. Using the previously published *L. chinense Actin97* as a reference gene (Tu et al., 2019), real-time quantitative PCR was performed. The results (Figure 4 [Figure 4: see original paper]) showed that all three AOX genes were expressed at significantly higher levels in floral organs than in vegetative organs. *LcAOX1a* exhibited extremely high expression in stamens, followed by petals, with minimal expression in stems, leaves, and leaf buds (approximately 1-fold). Similarly, *LcAOX1b* showed highest expression in stamens (about 2.69-fold higher than in leaves) and lowest in stems (about 0.2-fold of leaf expression). *LcAOX2* displayed highest expression in petals (approximately 3-fold higher than in leaves) and lowest in leaf buds.

Discussion

Alternative oxidase is a terminal oxidase of the alternative respiratory pathway, encoding a class of proteins controlled by AOX genes. Rhoads et al. (1991) first cloned an AOX gene from *Sauromatum venosum* and named it *AOX1.* Since then, numerous studies have investigated the alternative respiratory pathway and AOX genes. To date, AOX genes have been detected in almost all higher plant genomes, while in animals they are only found in a few lower organisms such as protozoa (McDonald et al., 2004). AOX genes have also been identified in fungi, bacteria, and algae (McDonald et al., 2006). The AOX gene family is a small nuclear-encoded gene family typically divided into two subfamilies: AOX1 and AOX2 (Selinski et al., 2018). Most monocots contain only the AOX1 subfamily, although AOX2 has been detected in ancient monocots such duckweed, anthurium, and devil' s ivy (Costa et al., 2017). Studies of stone pine

(*Pinus pinea*) revealed the presence of both AOX1 and AOX2 subfamilies, suggesting that the two subfamilies diverged before the separation of gymnosperms and angiosperms and that AOX2 was likely lost during subsequent evolution in most monocots (António et al., 2009). In dicots, both subfamilies are present except in a few species such as poplar (*Populus*), which contains only AOX1 (Zhang, 2014). Arabidopsis contains five AOX genes: four AOX1 genes (*AOX1a*, *AOX1b*, *AOX1c*, *AOX1d*) and one *AOX2* gene (Clifton et al., 2005; Saisho et al., 1997). Soybean contains one AOX1 gene and two AOX2 genes (*AOX2a* and *AOX2b*) (Thirkettle et al., 2003). Ito et al. (1997) isolated and identified two AOX genes from rice, designated *AOX1a* and *AOX1b*, and subsequent research identified *AOX1c* (Hiroaki et al., 2002), all belonging to the AOX1 subfamily. In this study, we identified and cloned three AOX family genes from *L. chinense* (*LcAOX1a*, *LcAOX1b*, and *LcAOX2*), and phylogenetic analysis confirmed their classification into AOX1 and AOX2 subfamilies.

According to the "SUM" model (Siedow & Umbach, 1995), AOX proteins contain one conserved "EXXH" motif in the first and fourth helices, forming a diiron carboxylate active center, and conserved "FXHR" and "EEE-Y" sequences at the C-terminus. The subsequent "AN" model proposed by Andersson & Nordlund (1999) suggested that AOX contains "EXXH" motifs in the second and fourth helices, with the active center located only in the fourth helix. Our protein homology analysis revealed that all three *L. chinense* AOX genes contained the characteristic "EXXH", "FXHR", and "EEE-Y" iron-binding conserved domains, with particularly high conservation at the C-terminus. Subcellular localization predictions indicated that LcAOX1a might be distributed outside mitochondria and chloroplasts, LcAOX1b in both chloroplasts and mitochondria, and LcAOX2 in mitochondria, consistent with reported experimental results for Arabidopsis AOX2, which plays an important role in the alternative respiratory pathway during seed germination (Saisho et al., 2001).

Tissue expression analysis revealed that *LcAOX1a*, *LcAOX1b*, and *LcAOX2* were all highly expressed in reproductive organs. Both *LcAOX1a* and *LcAOX1b* showed highest expression in stamens, with *LcAOX1a* being specifically and abundantly expressed in stamens at levels over 20,000-fold higher than in leaves. As an important mitochondrial terminal oxidase, AOX plays crucial roles in energy overflow and heat conversion. During flowering and pollination, floral organs may require heat to maintain active metabolic reactions, resulting in significantly enhanced AOX activity (Meeuse, 1975). The stamen-specific expression of *LcAOX1a* suggests that, in addition to abiotic stress responses, this gene may be closely associated with stamen development. Similar results have been reported in soybean, cotton (*Gossypium* spp.), and Arabidopsis, where AOX1 genes showed stamen-specific expression correlated with cytoplasmic male sterility (Shin et al., 2002; Li et al., 2013). Studies on cycad thermogenesis also indicated that male cones produced more heat than female cones, with unique mitochondrial morphology and CrAOX1-mediated microspore respiration potentially playing important roles in cone thermogenesis (Yasuko et al., 2019). Other studies suggest that AOX1 expression is not associated with cytoplasmic

male sterility but rather with microspore mother cell development (Johns et al., 1993). *LcAOX2* also showed higher expression in reproductive organs, particularly in petals, followed by calyx and pistil. Studies in watermelon and mango found that AOX2 was highly expressed during fruit development, suggesting a role in fruit development (Li et al., 2011; Considine, 2001). In Arabidopsis, *AOX2* showed highest expression in mature seeds (Nakabayashi et al., 2005), suggesting that AOX2 may be closely related to plant growth and development. This study cloned *L. chinense* AOX genes and explored the relationship between their tissue expression patterns and potential functions, providing a reference for future functional studies of AOX family genes.

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Note: Figure translations are in progress. See original paper for figures.