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Identification of Ascorbate Oxidase Genes and Their Response to Cold Stress in *Citrus sinensis*

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Abstract: Ascorbate oxidase (AAO) plays an important role in maintaining cellular redox homeostasis, thereby influencing plant growth, development, and responses to both biotic and abiotic stresses. However, there has been no systematic characterization of *AAO* genes in *Citrus*, especially their roles in response to cold stress. In the present study, nine *AAO* genes were identified in *C. sinensis* through bioinformatics analyses, exhibiting uneven distribution across four chromosomes. All CsAAOs possessed three conserved domains and were predicted to localize in the apoplast. The *CsAAO* gene family displayed varied intron–exon patterns. Phylogenetic analysis categorized the *CsAAO* family into three main clades (Clade A–C), suggesting distinct biological functions. Collinearity and Ka/Ks analysis revealed three duplicate gene pairs within the *CsAAO* gene family, with all duplicated CsAAOs primarily evolving under purifying selection. Analysis of *cis*-acting elements showed the presence of multiple hormone response elements and stress response elements within the *CsAAO* promoters. The computational analysis of microRNA target transcripts suggested that *CsAAO9* may be a target of csi-miR156. RNA-Seq data demonstrated high expression levels of *CsAAOs* in roots and young fruits, while qRT-PCR analysis showed significant upregulation of six *CsAAOs* in response to cold treatment. Furthermore, the activities of CsAAOs exhibited a pattern of initial decrease followed by an increase after exposure to low temperatures. These findings offer important insights into the role of *CsAAOs* in response to cold stress. Furthermore, *AAOs* could be target genes for breeding crops with better cold resistance.

Keywords: *Citrus sinensis*; ascorbate oxidase genes; gene family; cold stress; gene expression

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1. Introduction

Ascorbate oxidases (EC 1.10.3.3, AAOs) are classified as multi-copper oxidases and are found exclusively in plants and fungi [1]. AAOs are primarily located in the apoplast and their primary function is to facilitate the oxidation of ascorbate acid (AsA, reduced ascorbate) to monodehydroascorbate (MDHA) while simultaneously reducing molecular oxygen to water. MDHA undergoes spontaneous conversion to dehydroascorbate (DHA, oxidized ascorbate), which is then transported to the cytoplasm, where it is converted back to AsA through the ascorbate–glutathione (AsA-GSH) cycle [2]. Existing research indicates that AAOs play a crucial role in regulating the apoplastic redox state, thereby maintaining balanced levels of reactive oxygen species [3–5]. Additionally, AAOs may be involved in the processes of cell expansion and the loosening of cell walls in melon fruit [3,6]. Therefore, AAOs participate in the maintenance of cellular redox homeostasis, thereby exerting an influence on plant growth and development, as well as modulating responses to biotic

and abiotic stresses [4]. It is increasingly evident that *AAOs* are being considered potential candidates for enhancing crop stress tolerance [3,7].

Since AAOs were first described in cabbage leaf by Szent-Györgyi [8], it has been extensively investigated [1,3]. Early studies of AAOs primarily examined the enzyme's biochemical properties before expanding to explore its biological function. Of particular significance is the isolation of AAO cDNA from cucumber fruit tissues in 1989, leading to the subsequent cloning and identification of numerous *AAO* genes in various plant species [9–12]. In plants, AAOs are encoded by multiple genes, and some *AAO* gene family members have been identified and characterized in various plant species, including three members in *Arabidopsis*, five in rice, eight in cotton, four in maize, fourteen in bread wheat, seven in soybean, twelve in sugar beet, six in sorghum, and sixteen in *Ammopiptanthus nanus* [13–16]. Based on current understanding, the expression levels and enzymatic activity of *AAOs* exhibit notable variations across diverse plant species and tissues. For instance, Cucurbitaceae plants demonstrate elevated AAO activity in comparison to Solanaceae species. In tobacco, *AAO* genes are notably expressed in young and actively growing tissues, including the upper leaf, upper stem, and root [11]. In melon (*Cucumis melo*), the *CmAO4* gene is more abundant in vegetative tissues, whereas the *CmAO1* gene is predominantly expressed in fruit and floral tissues [17]. Additionally, in bread wheat (*Triticum aestivum*), the majority of *TaAAOs* exhibit high expression in root tissues [18]. These findings suggest that AAOs play crucial and varied functional roles across different tissue types.

The role of AAOs in environmental stress responses has garnered a growing interest, as AAOs serve as a key regulator of redox status in the apoplast. Typically, in transgenic plants, AAOs demonstrate a negative regulatory effect on drought and salt stress tolerance, as well as on disease resistance to fungal pathogens. For instance, in rice and sugar beet, the expression of *AAOs* decreased significantly under drought and salt treatments [13,15]. Notably, *AAO* antisense plants exhibited greater tolerance to drought or salt stress compared to wildtype plants in tomato [19,20]. Additionally, tobacco plants overexpressing *AAOs* showed increased susceptibility to fungal pathogen infection [21]. Interestingly, AAOs exhibit positive regulation of cold stress and viral resistance in transgenic plants. In *A. nanus*, the expression of three *AAO* genes was upregulated after seven days of cold stress, and overexpression of *AnAAO5* improved the cold stress tolerance of *Arabidopsis* plants [16]. In tobacco or rice, virus infection typically induces the expression of *AAOs*, while silencing *AAOs* has been shown to significantly diminish disease resistance [22,23]. This discrepancy may be attributed to alterations in ascorbic acid levels resulting from changes in *AAO* expression [3].

Citrus, particularly sweet orange (*C. sinensis*), is a significant and extensively cultivated fruit crop globally. Despite its widespread cultivation, low temperature stress significantly impacts the growth, development, and distribution of *Citrus*, posing challenges to the *Citrus* industry [24,25]. For example, the cold waves experienced in 2016 and 2021 led to differing levels of economic losses within China's citrus industry. Thus, the identification of key genes responsive to low temperatures is essential for the development of cold-resistant *Citrus* varieties [26–29]. While the *AAO* gene is known to play a crucial role in abiotic stress responses, its specific involvement in *Citrus* low-temperature stress remains unclear. In the present study, nine *AAO* genes were identified from the *C. sinensis* genome and their physicochemical properties, chromosomal localization, gene structures, phylogenetic relationships, duplication events, and *cis*-acting elements were analyzed. Subsequently, the expression profiles of *CsAAO* genes in various tissues and their response to cold stress were examined using public RNA-seq data and quantitative real-time PCR (qRT-PCR). Finally, the AAO activity of *C. sinensis* leaves was investigated after cold treatment. Overall, the findings of this study offer valuable insights for future functional analysis of *CsAAO* genes in the context of cold stress response.

2. Materials and Methods

2.1. Genome-Wide Identification of the AAO Gene Family in Citrus sinensis

For the purpose of identifying *Citrus AAO* genes, the protein sequences and genome of *Citrus* were obtained from the *Citrus* Pan-genome to Breeding Database. BLASTP analysis was conducted using three *Arabidopsis* AAO protein sequences sourced from The *Arabidopsis* Information Resource, along with the Hidden Markov Model (HMM) profile of the three Cu-oxidase domains (PF00394, PF07731, and PF07732) obtained from the pfam database as the queries. The putative CsAAOs were subsequently validated using the Pfam and SMART databases to identify conserved domains. The *CsAAOs* were designated *CsAAO1–9* based on their chromosomal location, with the chromosomal location map of these genes visualized using TBtools software (https://github.com/CJ-Chen/TBtools/ accessed on 6 March 2023) [30].

2.2. Physicochemical Properties Analysis

The molecular weight, isoelectric point, instability index, and grand average of hydropathicity of each CsAAO were predicted using the online software ExPASy (http://web.expasy.org/protparam/, accessed on 6 March 2023). The subcellular localization of CsAAOs was predicted by the online software CELLO (v.2.5, http://cello.life.nctu.edu.tw/, accessed on 6 March 2023) [31].

2.3. Gene Structure and Conserved Motif Analysis

The exon–intron distribution pattern of *CsAAO* genes was displayed based on the genome annotation file using TBtools software [30]. The MEME database (https://www.kaggle.com/datasets/utkarshx27/meme-database, accessed on 6 March 2023) was utilized to predict conserved motifs in CsAAO proteins, with a maximum number of 6 motifs and default parameters.

2.4. Phylogenetic Tree, Ka/Ks, and Collinearity Analysis

Phylogenetic trees were generated utilizing the Neighbor-joining algorithm in MEGA X software (https://www.megasoftware.net/, accessed 10 March 2023). The nucleotide substitution rates for nonsynonymous (Ka) and synonymous (Ks) mutations, as well as the Ka/Ks ratios of homologous *CsAAO* gene pairs, were determined using the simple Ka/Ks calculator in TBtools. Collinearity analysis between the genomes of *Citrus* and rice/*Arabidopsis* was conducted using the Multiple Systeny Plot in TBtools.

2.5. Prediction of Cis-Acting Elements and microRNAs Targeting CsAAO Genes

The *cis*-acting regulatory elements within the 2000 bp region upstream of the transcription start site of *CsAAOs* were predicted through the PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed 12 March 2023) [32]. The *Citrus* microRNA sequences retrieved from the miRBase database were subjected to analysis for miRNA target site presence in *CsAAOs* by the psRNATarget server [33], with stringent parameters set at a maximum expectation value of 3.5 and default settings for all other parameters.

2.6. Expression Profiles of CsLACs in Various Tissues

The FPKM values for *CsAAO* genes were downloaded from the *Citrus* Pan-genome to Breeding Database (http://citrus.hzau.edu.cn/, accessed on 12 May 2023). Heatmaps illustrating the expression profiles of *CsAAOs* were generated using TBtools.

2.7. Plant Materials and Low Temperature Treatments

Sweet orange plants (*C. sinensis*) were cultivated in a greenhouse under controlled conditions of 25 °C and 60% humidity, with a photoperiod of 16 h of light and 8 h of darkness. For the low temperature treatment, one-year-old sweet orange seedlings were subjected to an immediate temperature reduction to 4 °C for varying duration of 0, 2, 4, 6, 8, and 12 h. Following treatment, leaf samples were promptly frozen in liquid nitrogen and

stored at −80 °C until RNA extraction. AAO activities were assessed using commercially available assay kits (Shanghai Shangbao Biotechnology Ltd., Shanghai, China).

2.8. RNA Extraction and Expressing Profile Analysis

Total RNA was isolated utilizing RNA Isolation Reagent (Nanjing Nuoweizan Biotechnology Co., Ltd., Nanjing, China) according to the manufacturer's protocol. RNA quality was assessed through gel electrophoresis and a bioanalyzer (Agilent2100, Beijing, China). Subsequently, first strand cDNA synthesis was carried out using HiScript First Strand cDNA Synthesis Kit (Nanjing Nuoweizan Biotechnology Co., Ltd., China) following the manufacturer's guidelines. Data analysis was conducted with β-actin expression serving as a reference. The gene-specific primers utilized for qRT-PCR analysis are detailed in Supplementary Table S1. The qRT-PCR experiments were conducted using the CFX96 real-time PCR machine manufactured by BIO-RAD (Hercules, CA, USA). The reaction conditions and protocol for qPCR were consistent with those described in our previous publication [27]. The $2^{-\Delta\Delta CT}$ Ct method was employed to determine the relative gene expression levels across the samples [34]. For each experiment, three biological replicates were performed.

3. Results

3.1. Identification, Physicochemical Properties, and Chromosomal Location of the CsAAO Family

Based on BLASTP and HMMER searches, a total of nine candidate *CsAAO* genes were identified from *C. sinensis* genome (v3.0). These putative *CsAAOs* were designated as *CsAAO1* to *CsAAO9* according to their chromosomal positioning. As shown in Figure 1, nine *CsAAO* genes were randomly anchored on four chromosomes (chr1, chr2, chr3, and chr8). The physicochemical characteristics of *CsAAO* genes were analyzed, revealing variations in the amino acid length of CsAAO proteins ranging from 538 (CsAAO5) to 586 (CsAAO1) and calculated molecular weights from 59.52 kDa to 66.16 kDa (Table 1). The isoelectric point values fell within a range of 7.37 (CsAAO6) and 9.37 (CsAAO4). Among the CsAAO proteins, only CsAAO1 and CsAAO4 exhibited instability, with instability index (II) values exceeding 40. The grand average of hydropathicity (GRAVY) values for all CsAAO proteins was negative, indicating hydrophilicity. Additionally, subcellular localization prediction using CELLO v.2.5 software suggested that all CsAAO proteins were extracellularly localized.

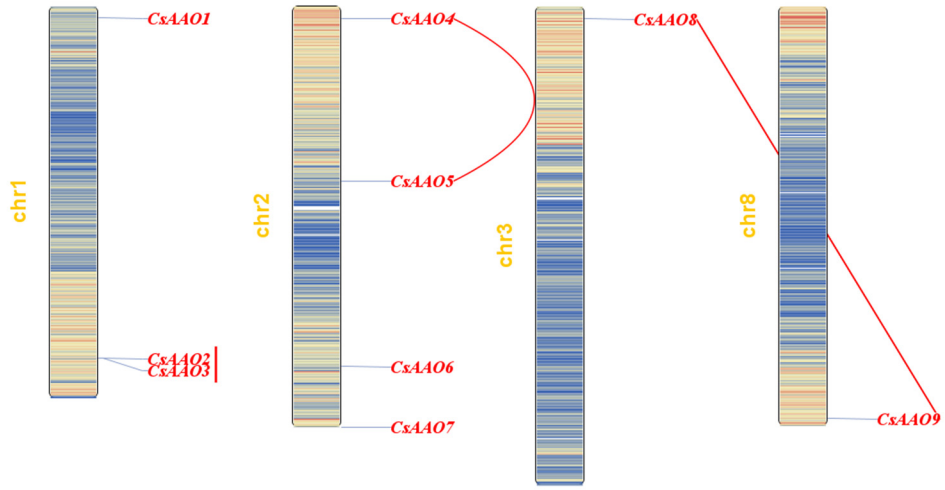

Figure 1. Chromosomal distribution of *CsAAO* gene family members. Pairs of segmentally duplicated genes are indicated by red lines.

Table 1. Basic information for the *CsAAO* genes in the *C. sinensis* genome.

Gene Name	Gene ID	Amino Acids Length	Molecular Weight (kDa)	Isoelectric Point (pI)	Instability Index (II)	Grand Average of Hydropathicity (GRAVY)	Subcellular Localization Predicted
CsAAO1	Cs_ont_1g000870.1	586	66.16	8.78	40.14	−0.266	Extracell
CsAAO2	Cs_ont_1g025500.1	539	60.29	9.17	34.78	−0.199	Extracell
CsAAO3	Cs_ont_1g025510.1	541	60.48	9.15	39.82	−0.164	Extracell
CsAAO4	Cs_ont_2g001690.1	543	61.47	9.73	43.86	−0.259	Extracell
CsAAO5	Cs_ont_2g018990.1	538	59.52	8.09	38.34	−0.227	Extracell
CsAAO6	Cs_ont_2g030090.1	580	64.72	7.37	32.56	−0.318	Extracell
CsAAO7	Cs_ont_2g035780.1	554	62.19	8.27	32.50	−0.252	Extracell
CsAAO8	Cs_ont_3g001440.1	542	60.21	9.27	30.76	−0.227	Extracell
CsAAO9	Cs_ont_8g028600.1	545	60.91	9.32	37.26	−0.234	Extracell

3.2. Analysis of Conserved Motifs, Domain Composition, and Gene Structure of CsAAOs

The comparison of conserved motifs, domain composition, and gene structure among members of a gene family is widely recognized as a valuable approach for gaining insights into their functional characteristics. In the present study, six conserved motifs were identified in CsAAO proteins, as illustrated in Figure 2. It is noteworthy that CsAAO1 and CsAAO6 within the same group were found to lack motif 4 (Supplementary Figure S1), whereas all other CsAAO proteins exhibited the presence of all six motifs (Figure 2a,b). Additionally, Figure 2c demonstrates the presence of Cu-oxidase, Cu-oxidase_2, and Cu-oxidase_3 domains in all CsAAO proteins. Examination of the gene structures of *CsAAO* revealed variations in the number of exons, ranging from 2 to 8 (Figure 2d). Interestingly, the size of *CsAAO* genes appeared to be influenced by the size of introns. In summary, certain *CsAAO* genes within the same group exhibit similar gene structures.

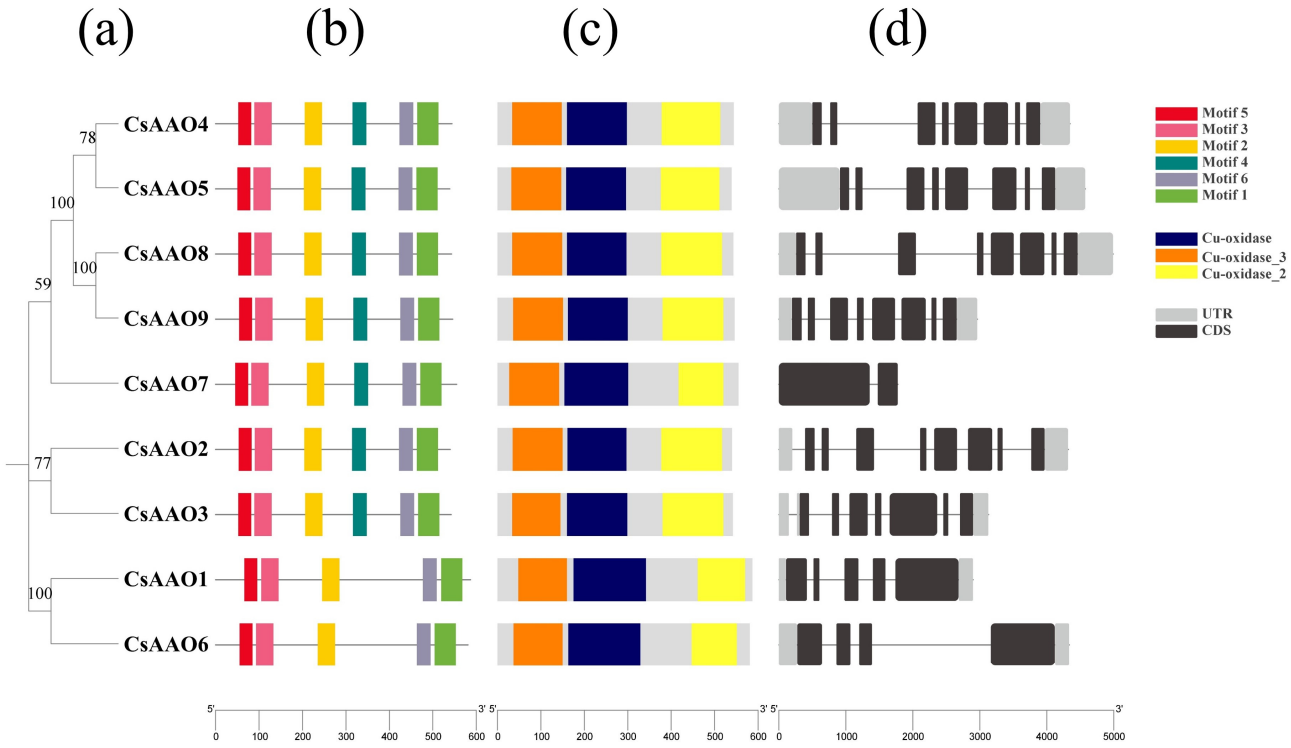

Figure 2. Phylogenetic trees, conserved motifs, conserved domains, and gene structures of CsAAOs in the *C. sinensis*. (**a**) The phylogenetic tree was generated by MEGA-X software using the Neighbor-joining (NJ) method. (**b**) Six conserved motifs of CsAAO proteins. (**c**) Conserved motifs of CsAAO proteins. (**d**) Gene structures of *CsAAOs*.

3.3. Phylogenetic Analysis of AAO Proteins in Different Species

Phylogenetic analysis was conducted using 17 AAO predicted proteins from *Citrus*, *Arabidopsis*, and rice to construct an evolutionary tree (Figure 3). The resulting phylogenetic tree revealed that the AAOs were grouped into three distinct clades, with CsAAOs present in each clade. Specifically, CsAAO6 was assigned to Clade A, CsAAO1 to Clade B, and CsAAO2, CsAAO3, CsAAO4, CsAAO5, CsAAO7, CsAAO8, and CsAAO9 to Clade C.

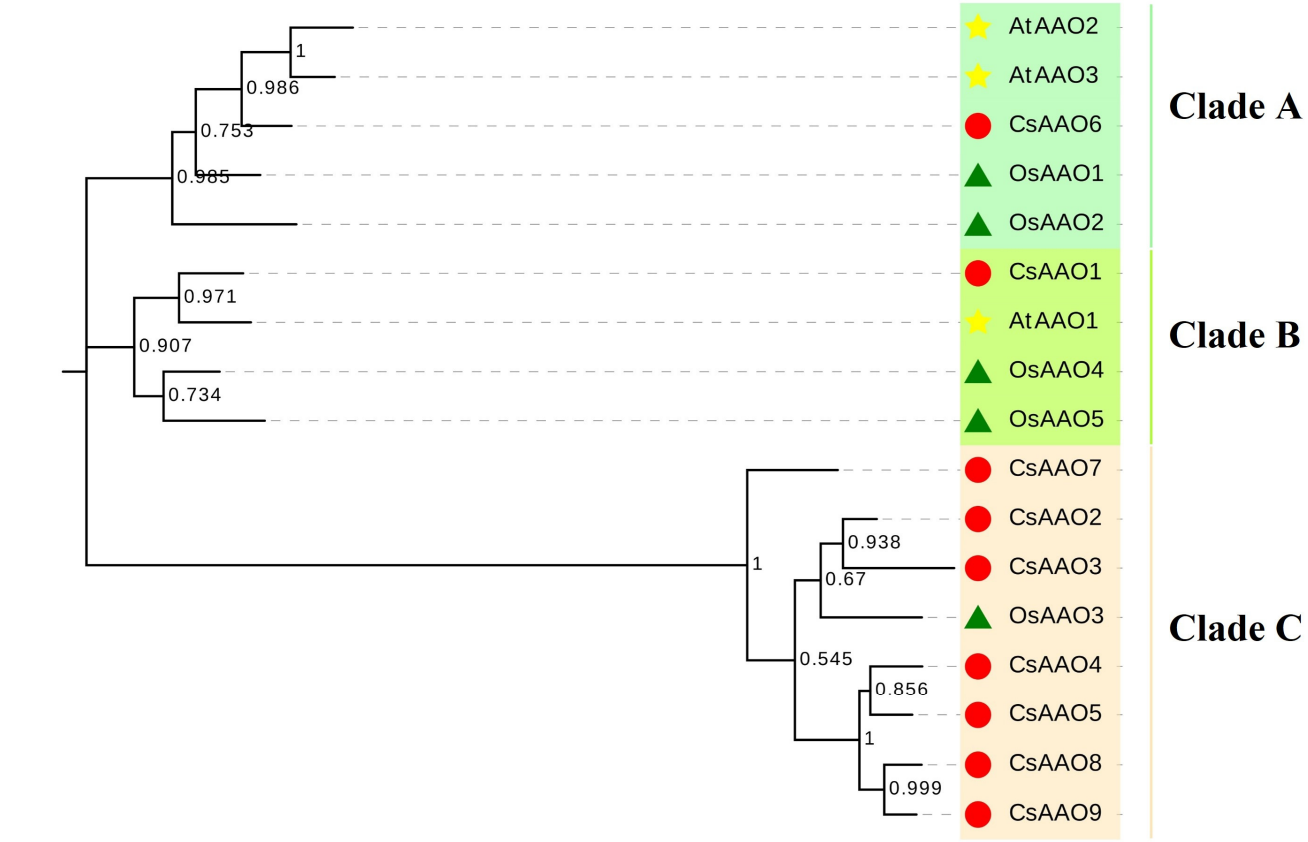

Figure 3. Phylogeny of the AAOs in *C. sinensis*, *Oryza sativa*, and *Arabidopsis thaliana*. The phylogenetic tree was generated utilizing the Neighbor-joining method with p-distance substitution model (gamma = 1) and 1000 bootstrap replicates in MEGA-X software.

3.4. Gene Duplication and Genome Synteny Analysis of AAO Family Genes

In order to examine the gene duplication events of the *AAO* family, a collinearity analysis was conducted. Within the *CsAAO* gene family, the analysis revealed the presence of two segmental duplication gene pairs (*CsAAO4*—*CsAAO5*, *CsAAO8*—*CsAAO9*) and one tandem duplication gene pair (*CsAAO2*—*CsAAO3*) within the *C. sinensis* genome (Figure 1), suggesting that gene duplication events have facilitated the expansion and evolution of the *CsAAO* family. Additionally, the Ka/Ks ratio for each duplicated gene pair was found to be less than 0.5, indicating that all duplicated genes have been subjected to strong purifying selection (Table 2). Comparing the *CsAAO* family with rice and *Arabidopsis AAO* family revealed a high homology between *C. sinensis* and Arabidopsis genes, suggesting similar functions. The collinear region between *C. sinensis* and *Arabidopsis* is greater than that with rice, indicating closer orthologs between *C. sinensis* and *Arabidopsis* (Figure 4).

Table 2. The Ka/Ks ratios for duplicate *AAO* genes in *C. sinensis*.

Paralogous Genes	Ka	Ks	Ka/Ks	Selective Pressure
CsAAO2—CsAAO3	0.1689	1.6646	0.1015	Purifying selection
CsAAO4—CsAAO5	0.1464	1.3449	0.1089	Purifying selection
CsAAO8—CsAAO9	0.2440	1.4973	0.1629	Purifying selection

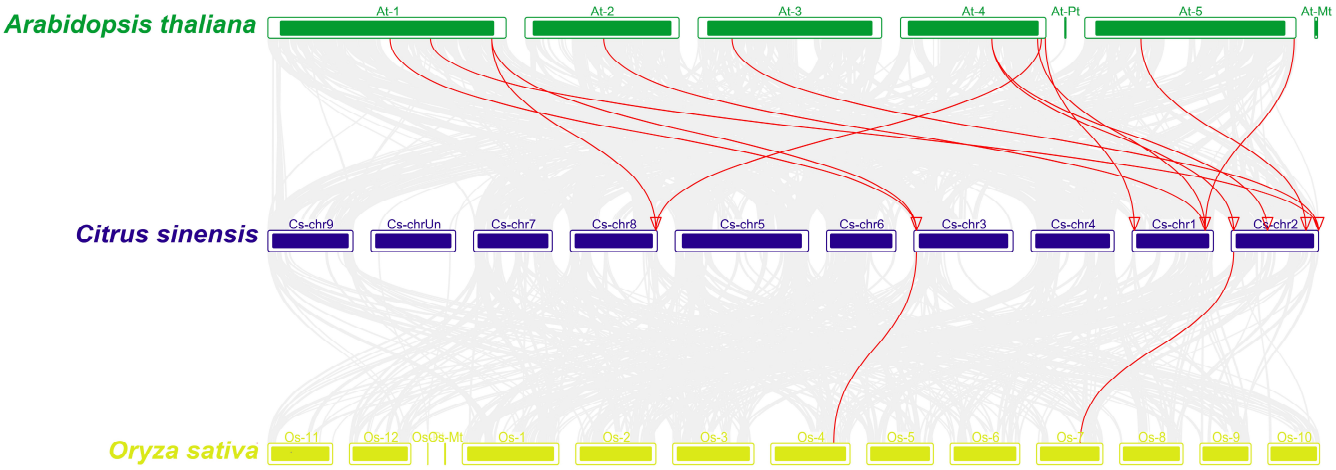

Figure 4. Genome-wide synteny analysis for *AAO* genes among *C. sinensis*, *Oryza sativa*, and *Arabidopsis thaliana*.

3.5. Cis-Acting Element and MicroRNA Target Site Analysis of CsAAOs

Cis-acting elements have been identified as crucial components in the control of gene expression [35]. In order to gain a deeper insight into the function of *CsAAO* genes and the mechanisms of transcriptional regulation, the *cis*-acting elements located within the 2000 base pair upstream regions of the translation initiation site were analyzed for nine *CsAAO* genes utilizing the PlantCARE software (Figure 5). The examination of *cis*-acting elements indicated that the promoter regions of *CsAAO* genes encompass 39 distinct types of *cis*-acting elements, which can be classified into three primary categories: development elements, hormone response elements, and stress response elements. Notably, stress response elements were found to be the most prevalent, comprising a total of 25 occurrences. Development elements primarily include meristem-specific elements (CAT-box), circadian regulation elements (circadian), and endosperm expression regulatory elements (GCN4_motif). Hormone response elements mainly consist of abscisic acid-responsive elements (ABREs), methyl jasmonate-responsive elements (CGTCA-motif), and salicylic acid-responsive elements (TCA-elements). Stress response elements mainly encompass light-responsive elements (Box 4, G-Box, etc.), anaerobic induction elements (AREs), and low-temperature-responsive (LTR) elements. Of note, the promoter region of some *CsAAO* genes contains unique *cis*-acting elements, such as wound-responsive elements (WUN-motif) in the *CsAAO1* promoter and gibberellin-responsive elements (TATC-box) in the *CsAAO6* promoter.

MicroRNAs (miRNAs) are a group of small endogenous non-coding RNAs, typically 20–24 nucleotides in length, known for their ability to downregulate gene expression post-transcriptionally through mechanisms such as direct transcript cleavage or translational inhibition. This regulatory function of miRNAs has been shown to impact various aspects of plant biology, including growth, development, and response to stress [36]. In this study, all miRNAs identified in *Citrus* were utilized as query sequences to predict potential target sites on *CsAAO* genes using the psRNATarget tool with stringent parameters. The analysis revealed that two specific *Citrus* miRNAs (csi-miR156h/i) are likely regulators of the *CsAAO9* gene (Table 3).

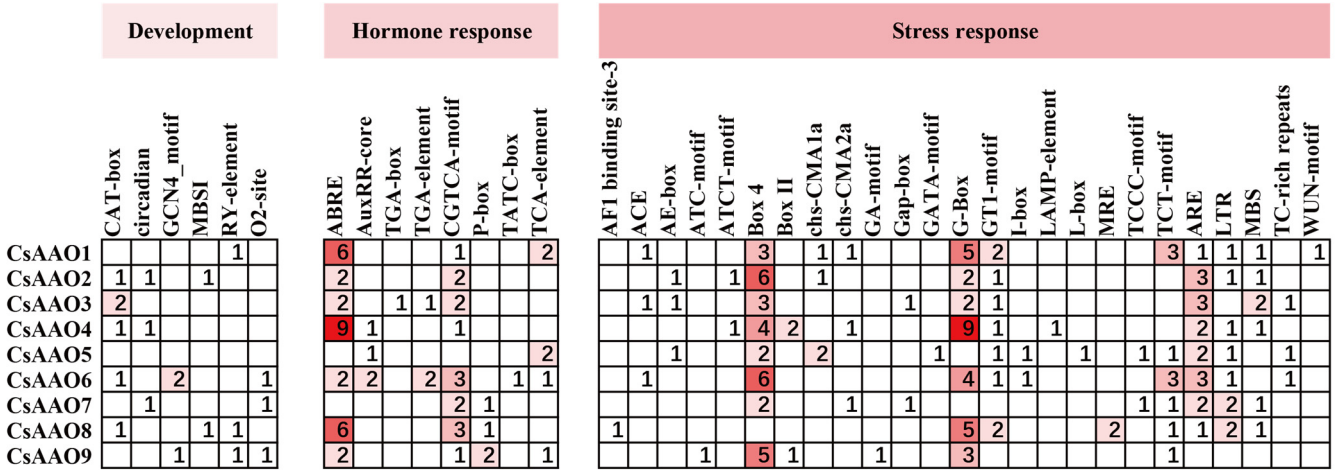

Figure 5. The distribution of *Cis*-acting elements in promoters of *CsAAO* genes.

Table 3. Prediction of microRNA-targeting *CsAAO* genes.

Gene Name	Predicted miRNA Target Sites	miRNA Length	Expectation	Inhibition
CsAAO9	csi-miR156h	21	3.5	Cleavage
CsAAO9	csi-miR156i	21	3.5	Cleavage

3.6. Gene Expression Analysis of CsAAOs in Different Tissues

In order to elucidate the potential functions of *CsAAO* genes, an analysis was conducted on the available RNA-seq data from the *Citrus* Pan-genome to Breeding Database. The findings revealed that *CsAAO* gene expression displays tissue specificity, with *CsAAO2* and *CsAAO6* exhibiting notably high expression levels in various tested tissues such as leaves, seeds, callus, roots, early-stage ovules, late-stage ovules, young fruit flesh, and mature fruit flesh (Figure 6). Conversely, *CsAAO7* was not detected in any of the eight tissues examined. Noteworthy is the observation that *CsAAO3* was exclusively expressed in roots. Overall, the nine *CsAAO* genes demonstrated higher expression levels in roots and young fruit tissues.

3.7. Analysis of CsAAOs Expression Patterns and Enzymatic Activities under Cold Stress

The *AAO* gene family is known to be involved in abiotic stress responses. Therefore, quantitative real-time polymerase chain reaction (qRT-PCR) was conducted to assess the transcript levels of *AAO* in response to low temperature treatment in order to investigate the ability of the *CsAAO* family to respond to cold stress. As depicted in Figure 7, the expression patterns of the *CsAAO1*, *CsAAO3*, *CsAAO5*, and *CsAAO7* genes exhibit an initial increase followed by a decrease, whereas the *CsAAO2*, *CsAAO4*, *CsAAO6*, and *CsAAO8* genes demonstrate a decrease followed by an increase. *CsAAOs* exhibiting a marked decline in expression from 0 to 2 h may experience a transient transcriptional repression as a consequence of the cold shock. The expression of the *CsAAO9* gene shows a consistent upward trend. Noteworthy is the substantial upregulation of *CsAAO1* by 42.4-fold at 2 h, *CsAAO7* by 130.9-fold at 8 h, and *CsAAO9* by 47.3-fold at 12 h compared to the control group. The observations revealed that *CsAAO1*, *CsAAO5*, *CsAAO7*, and *CsAAO9* were implicated in the cold stress response of *C. sinensis*. Additionally, the activities of the CsAAOs demonstrated a pattern of initial decline, followed by an increase upon exposure to low temperatures (Figure 8).

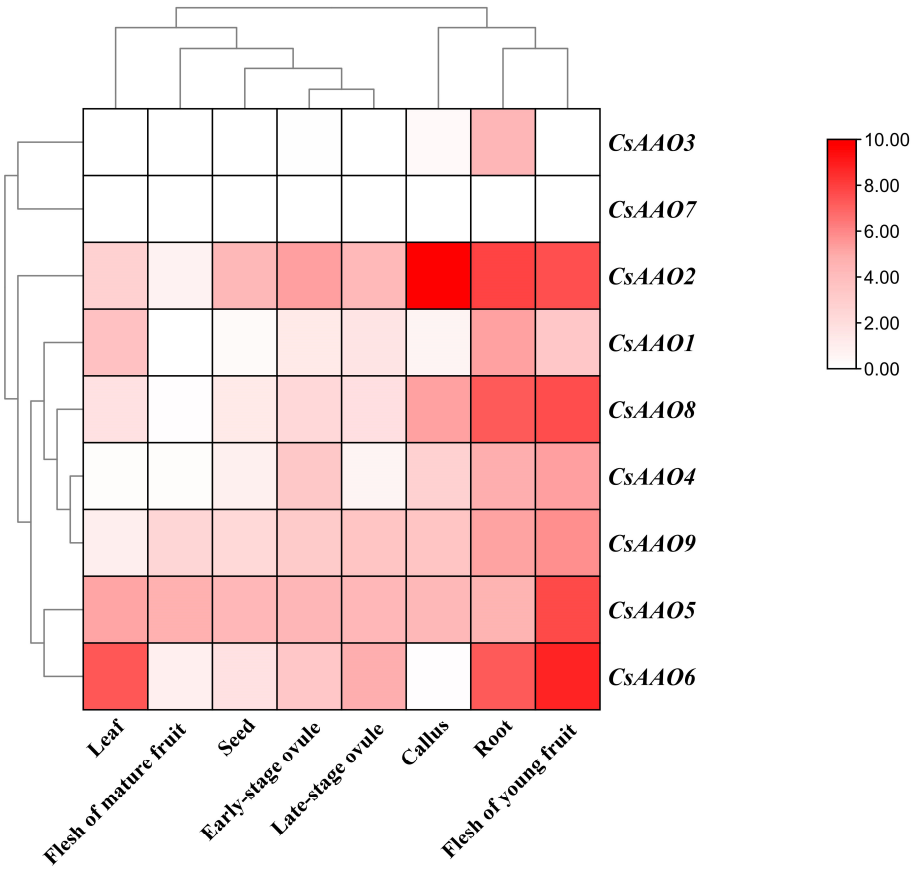

Figure 6. The spatial expression patterns of the *CsAAO* genes.

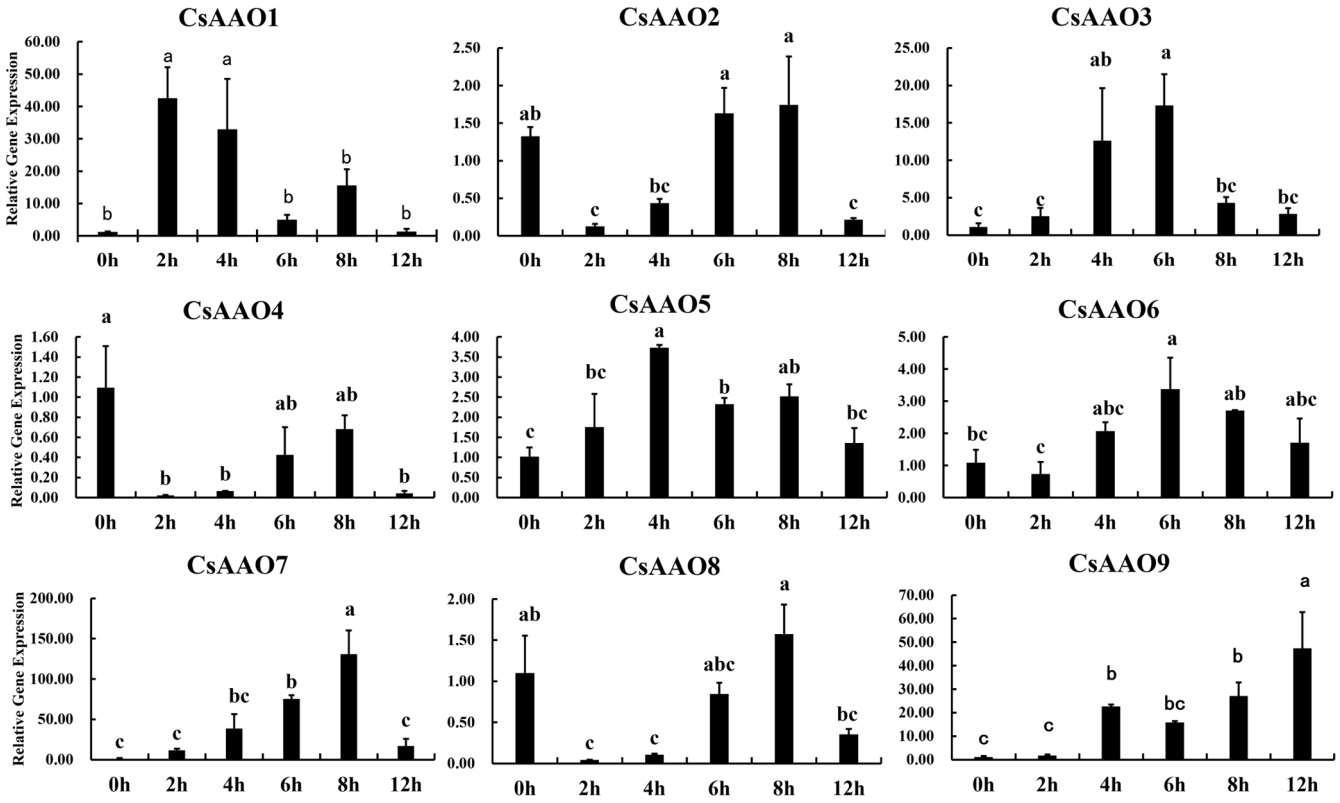

Figure 7. Expression profiles of the *CsAAO* genes in response to cold treatment. Different letters indicate significant differences (ANOVA analysis, $p < 0.05$).

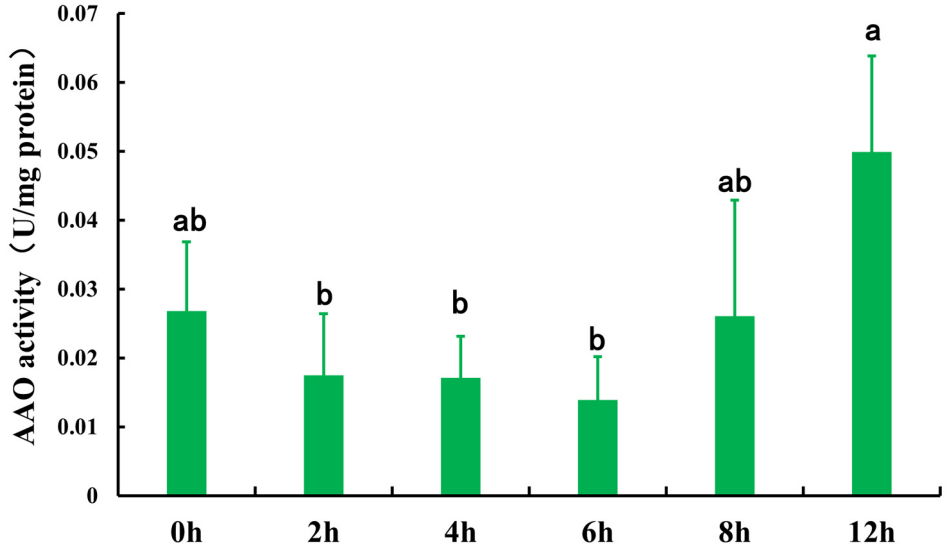

Figure 8. Changes in CsAAO activity under low-temperature treatments. Different letters indicate significant differences (ANOVA analysis, $p < 0.05$).

4. Discussion

In the current work, we identified nine *AAO* genes from *C. sinensis* by bioinformatics analyses. The number of *AAO* gene family members varies from 3 to 16 among different plant species [13–16]. After analyzing the *AAO* gene family members across various species and their respective genome sizes, it was determined that there is no significant correlation between genome size and the number of *AAO* gene family members [18]. Additionally, there is no significant difference in the number of *AAO* gene members between monocotyledonous and dicotyledonous plants. Subcellular localization predicted that all CsAAO proteins were localized in the apoplast (Table 1). These findings align with previous research [16,18], indicating the proteins' potential involvement in maintaining cellular redox homeostasis. Examination of conserved motifs, domain composition, and gene structure of CsAAOs revealed that the *CsAAO* gene family exhibited a notable degree of conservation throughout the course of evolution (Figure 2). Notably, the conserved domains/motifs within the *AAO* gene family members exhibit high similarity in five plant species including rice, *Arabidopsis*, soybean, maize, and sorghum [13]. Thereby, the *AAO* gene family may be an ancient and evolutionarily conserved gene family in plants.

To compare the function and evolutionary relationships of CsAAO proteins in dicots and monocots, amino acid sequences from rice, *Arabidopsis*, and *C. sinensis* AAOs were used to create a phylogenetic tree (Figure 3). The results showed that AAOs from *C. sinensis*, *Arabidopsis*, and rice grouped into three clades, supporting previous findings [13,14]. The presence of the same clades in all three species suggests that *AAO* genes existed before the split between monocots and dicots. Furthermore, it was observed that seven CsAAOs from *C. sinensis* and OsAAO3 from the monocot model plant rice were clustered together in the same phylogenetic branch. The *OsAAO3* gene was responsive to salinity and drought stresses [13]. Consequently, it is hypothesized that the seven *CsAAOs* may share a similar function with *OsAAO3*. However, additional research is required to validate this assertion.

Analysis of gene collinearity within and between species offers valuable information on genome evolution, conservation of gene function, and evolutionary connections [37]. In this study, we have identified three putative *C. sinensis* paralogs (Cs-Cs), thirteen orthologs shared between *C. sinensis* and *Arabidopsis thaliana* (Cs-At), and two orthologs shared between *C. sinensis* and *Oryza sativa* (Cs-Os) (Figures 1 and 4). In *Glycine max*, three *AAOs* duplicated tandemly and four *AAOs* duplicated segmentally [13]; in *Ammopiptanthus nanus*, four *AAOs* duplicated tandemly and eight *AAOs* duplicated segmentally [16]; in *Gossypium hirsutum*, two *AAOs* duplicated tandemly and six *AAOs* duplicated segmentally [14]. These findings suggest that gene duplication events, particularly segmental

duplication, played a significant role in the evolutionary development and proliferation of the *AAO* gene family in plant species [38]. Additionally, Ka/Ks analysis indicated that the duplicated genes of *CsAAOs* may have been subjected to significant purifying selection pressure (Table 2), a finding consistent with previous studies on *Ammopiptanthus nanus* and *Gossypium hirsutum* [14,16].

MicroRNAs have been demonstrated to be significant regulators of gene expression post-transcriptionally. A recent study identified miR528 as a negative regulator of viral resistance in rice by targeting *AAO* messenger RNA [23]. Additionally, miR4415 was predicted to target *AnAO5* and *AnAO11* in *Ammopiptanthus nanus* [16]. Our findings suggest that csi-miR156 may target *CsAAO9*, although experimental validation is required to confirm this prediction.

PlantCARE analysis revealed multiple *cis*-acting elements in the promoters of *CsAAO* genes, categorized into development, hormone response, and stress response elements (Figure 5). This distribution pattern is consistent with other plant species [14,16], suggesting that *AAO* genes play a role in multifarious physiological procedures. The predominant *cis*-acting elements identified in this study were stress response elements, with a total of 25 instances observed. The stress response elements include light-, anaerobic induction-, low-temperature-, drought-, wound-, and defense/stress-responsive elements. These *cis*-acting elements help *CsAAOs* respond to oxidative stress from adverse conditions, aiding in maintaining extracellular redox balance. The promoter regions of all *CsAAO* genes, with the exception of *CsAAO9*, contain anaerobic induction elements. It is well established that anaerobic induction elements are typically activated in roots following water excess and during seed germination [39]. Our observations further indicate that the majority of *CsAAOs* exhibit high expression levels in roots (Figure 6). These findings suggest that *CsAAOs* may play a role in conferring anaerobic tolerance in roots. Notably, the promoter regions of certain *CsAAO* genes have unique *cis*-acting elements, like wound-responsive elements (WUN-motif) in *CsAAO1* and gibberellin-responsive elements (TATC-box) in *CsAAO6*. We hypothesize that other plant AAOs within the same phylogenetic subgroup as CsAAO1 and CsAAO6 may similarly contain these specific *cis*-acting elements. Of particular interest, the LTR motif was detected in the promoter regions of seven *CsAAOs* (*CsAAO1-2* and *CsAAO4-8*). Subsequently, the responses of the *CsAAOs* to cold stress were examined through qRT-PCR analysis (Figure 7). Remarkably, differential expression of the seven *CsAAOs* was observed at various time points following cold treatment, with *CsAAO7* demonstrating the most pronounced upregulation (130.9-fold at 8 h). Unexpectedly, *CsAAO7* expression was not detected in any of the eight tissues analyzed. Nevertheless, *CsAAO7* expression was observed at very low levels in newly induced calluses and mature leaves (Supplementary Table S2), as well as in mesophyll protoplasts (our unpublished data). We hypothesize that *CsAAO7* expression is regulated by cold treatment. Furthermore, AAO activity peaked after 12 h of exposure to low temperatures (Figure 8). Further investigation is warranted to elucidate the precise physiological function of this gene. In *Ammopiptanthus nanus*, *AnAO5* was found to be involved in the low-temperature response, with low-temperature response *cis*-elements present in its promoter regions [16]. Subsequent functional analyses demonstrated that the overexpression of *AnAO5* resulted in increased cold stress tolerance in *Arabidopsis* seedlings. These findings suggest a potentially important function of the *AAO* gene in the response of plants to cold stress, particularly in relation to low-temperature-response-related *cis*-elements.

5. Conclusions

Nine *AAO* genes were found in *C. sinensis*, with uneven distribution across four chromosomes. All CsAAOs have three conserved domains and are predicted to localize in the apoplast. The *CsAAO* gene family has diverse intron–exon patterns and is divided into three main clades. Three duplicate gene pairs were identified, evolving under purifying selection. *Cis*-acting elements in *CsAAO* promoters include hormone and stress response elements. Computational analysis suggests *CsAAO9* may be targeted by csi-miR156. *CsAAOs*

are highly expressed in roots and young fruits, with six *CsAAOs* upregulated in response to cold treatment. CsAAO activities initially decrease then increase after exposure to low temperatures. Our findings indicate that the *CsAAO* genes may play a role in the response to cold stress and could serve as potential target genes for breeding agricultural crops with enhanced cold resistance.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agriculture14091643/s1, Figure S1: Sequence logos of conserved motifs in CsAAOs, Table S1: Primers used in this study, Table S2: The expression patterns of CsAAO7 in the Citrus Pan-genome to Breeding Database.

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