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GENOME-WIDE IDENTIFICATION AND EXPRESSION ANALYSIS OF *AAO* GENE FAMILY IN MAIZE

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Abstract

Ascorbic acid oxidase (AAO) is a member of the multi-copper oxidase family and plays an important role in plant growth and development. The functional characteristics and expression patterns of the *AAO* gene family were analyzed using the maize (*Zea mays* L.) B73 genomic mRNA and amino acid database. Bioinformatics analysis of the whole genome *AAO* gene of maize had identified 36 *AAO* gene members, and predictive analysis was performed on the chromosomal location, the mode and time of gene differentiation, protein physicochemical properties and secondary structure, gene structure, conserved elements, phylogenetic tree and expression patterns of these genes. The results showed that 36 *ZmAAO* were unevenly distributed on 10 chromosomes. The genes differentiation was dominated by fragment replication, and the differentiation time was about 3.3-25.75 million years. ZmAAO proteins were mostly basic proteins. The protein secondary structure is mainly composed of irregular curls. The analysis of gene structure and conserved motifs showed that there was no significant difference in the number of introns in *ZmAAO* genes, and the amino acid sequence was highly conserved. The phylogenetic trees can be divided into 4 subfamilies, in which maize, rice and sorghum has a large number of orthologous genes and close genetic relationships. The expression pattern analysis found that the expression level of *ZmAAO* was different in different tissues, which indicates that it functions differently. After high temperature stress, the expression level of *ZmAAO* gene was significantly different in different tissues, indicating that some *ZmAAO* genes respond to high temperature stress. These results provide a theoretical basis for studying the biological function of this gene family and breeding high temperature resistant varieties in the future.

Key words: Maize (*Zea mays* L.), *AAO* gene family, Bioinformatics analysis.

Introduction

Global climate change has a huge impact on food production, and especially high temperatures can severely hinder and damage plant growth and development (Fang & Xiong, 2015). According to the Global Climate Report of the National Oceanic and Atmospheric Administration (https://climate.nasa.gov/vital-signs/global-temperature/), the surface temperature of the earth in 2018 was 0.8℃ higher than that of the 20th century. Crop (including *Oryza sativa*, *Triticum aestivum*, and maize) production was declining as the rising global temperature (Lobell *et al.*, 2011; Bita & Gerats, 2013). High temperature stress will continue to occur in the future, so it is an urgent work to improve the tolerance of crops to high temperature (Horton *et al.*, 2015).

At present, maize is the second largest food crop in the world. As a C4-type crop, maize has great potential for production and is likely to become the largest food crop in the future (Jones, 2009; Ort & Long, 2014). As a temperature-loving crop, maize generally actively initiative to adapts to high temperatures, but when the temperature exceeds 35℃, it will adversely affect the growth and development of maize, such as thinning leaves, reducing carbon dioxide assimilation, the decrease of photosynthetic rate, the abortion of pollen, and the significant reduction of the number of grains per ear, the reduction of grain weight, and ultimately reduced kernel yield (Ren *et al.*, 2019). High temperature stress will also affect the physiological activities of maize, resulting in a large amount of active oxygen in the body, a membrane lipid peroxidation reaction and a significant decrease in root vitality (Sun *et al.*, 2017; Yu *et al.*, 2017). The ascorbate-glutathione cycle (ASA-GSH) is an important antioxidant pathway for scavenging ROS (Dong *et al.*, 2018). Ascorbic acid oxidase (AAO) is a class of oxidases exist in plants and some fungi, belonging to the blue copper oxidase family (Sanmartin *et al.*, 2007; Meng *et al.*, 2018), and also the one of the key enzymes in ASA-GSH circulation system. AAO catalyzes the oxidation of ASA to unstable monodehydroascorbic acid (MDHA). Due to the unstable nature of MDHA, it can be decomposed into dehydroascorbic acid, thereby regulating the redox homeostasis of ascorbic acid pools in plant exosomes (Shi *et al.*, 2008), which plays an important role in improving the abiotic stress resistance of plants.

Sequencing of whole maize genome provides a more convenient and effective method for identifying and mining important functional genes from the genome level by using bioinformatics (Jiao *et al.*, 2017; Lu *et al.*, 2015). This study uses bioinformatics to predict the subcellular location, gene structure, and physicochemical properties of *ZmAAO* gene family members, providing a theoretical basis for further study of the biological function of the gene family and breeding high temperature resistant varieties.

Materials and Methods

Search and identification of *ZmAAO* gene family: Taking maize as the research object, the genomic sequence, mRNA and protein sequences were obtained from the maize B73 genomic database (www.ncbi.nlm.nih.gov/genome/?term=Zea +mays+). Bioedit software was used to establish a local database of the entire maize genome amino acid sequence. HMM (Hidden Markov Model) profile of *AAO* domain (PF00394) was used as a query to run blast against the local database of the whole maize cDNA sequence by using the TBlastN (E-value =0.001), and the candidate gene sequence of *AAO* was preliminarily screened out. Then go to the corresponding

protein database to find the corresponding amino acid sequence, and then use the corresponding amino acid sequence of these gene sequences to blast against Pfam (http: // pfam. Wust.l edu / hmmsearch. Shtml) and CDD (www.ncbi.nlm. nih.gov/ Structure / cdd / wrpsb.cgi) database to check if it contains *AAO* domain. Remove the sequence that does not contain the multi-copper oxidase domain; use DNAMAN to arrange the amino acid sequence of the selected *AAO* domain in multiple sequences to remove duplicate sequences in candidate genes; and locate the *ZmAAO* gene by the TBtools (Chen *et al.*, 2018). "Chr N" (N is the chromosome number valued as 1~10) indicates that the sequence was located on the corresponding chromosome.

Duplication and *Ka / Ks* value analysis of *ZmAAO* gene: The phylogenetic tree of the *ZmAAO* gene family was constructed by the neighbor-joining (NJ) method with MEGA-X (Kumar *et al.*, 2018); then MCScanx software was used to analyze the replication relationship between genes (Wang *et al.*, 2012), and TBtools were used to calculate the non synonymous substitution rate (*Ka*) and the synonymous substitution rate (Ks) (Chen *et al.*, 2018); generally speaking, *Ka / Ks* <1 means the purification selection, *Ka / Ks* =1 means the neutral selection, and *Ka / Ks*>1 means the positive selection (Rozas, 2009; Librado & Rozas, 2009); the differentiation time was determined by the formula calculation as T = *Ks* / 2λ(λ= 6.5×10^{-9}) (Zhang *et al.*, 2011; Peng *et al.*, 2013).

Structure analysis of ZmAAO protein: Pretparam online software provided by ExPaSy (http://www. cn.expasy.org/tools) was used to predict and analyze the physical and chemical properties of ZmAAO protein (Imran & Liu, 2016); WOLFPSORT (https://wolfpsort. hgc.jp/) was used to perform subcellular localization of its protein sequence; SOPMA (https://npsa -prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_ sopma.html) predict and analyze the secondary structure of ZmAAO protein; SignalP4.0 Sever (http://www.cbs.dtu.dk/services/SignalP -4.0 /) was used for prediction analysis of signal peptide.

Analysis on structure and conserved motifs of *ZmAAO* gene: The genomic sequence, CDS, and genomic location information of *ZmAAO* were obtained from the NCBI maize database, and submitted to the Gene Structure DisplayServer (GSDS) (gsds.cbi.pku.edu.cn) online software to draw the exon-intron structure diagram (Guo *et al.*, 2007); MEME software (http://meme-suite.org/) was used to perform predictive analysis on the motif of *ZmAAO* (Ali *et al.*, 2017; Bailey & Elkan, 1995). The total number of motif was set to 20, and other parameters were default.

Phylogenetic analysis of ZmAAO protein: In order to study the evolutionary relationship between ZmAAO and other species, this study used *ZmAAO1* as a probe to search the homologous sequences of *Sorghum bicolor*, *Oryza sativa*, *Arabidopsis thaliana*, to check if it contains an AAO protein domain by passing through Smart (smart.embl-heidelberg.de/) and CDD (www.ncbi.nlm. nih.gov / Structure / cdd / wrpsb.cgi) program. Finally, the obtained AAO homologous sequence was submitted to MEGA-X for alignment, and then a neighbor-joining method (NJ) was used to construct the phylogenetic tree. The Bootstrap was repeatedly set to 1000 (Kumar *et al.*, 2018).

Expression analysis of *ZmAAO* gene: Transcriptome data of *ZmAAO* gene expression in different tissues (root, stalk, leaf, silk, ear,tassel) of different development stages (3-leaf stage and pollen dispersal stage) at different high temperature stressing time (38℃, 0h, 2h, 48h) for maize was downloaded from NCBI SRA database (https://www. ncbi.nlm.nih.gov/sra), with accession number as PRJNA520822 (He *et al.*, 2019); the R language ggplot2 toolkit was used to draw a gene expression heat map.

Results and Analysis

Identification and analysis of chromosomal location of *ZmAAO* gene family: TBlastN was used to blast against the whole genome genes of maize with the amino acid sequence of *AAO* domain to obtain candidate *AAO* genes. Then, the multiple sequence alignment analysis was performed to remove redundant genes, and the *AAO* genes contained the *AAO* domain were selected through Pfam database. A total of 36 *AAO* family genes members were identified in maize genome database. They were named from top to bottom based on their position on the chromosome (Table 1) (Wei & Pan, 2014). The distribution characteristics of *ZmAAO* on chromosomes were analyzed by Tbtools. The results showed that *36 AAO* genes were unevenly distributed on 10 chromosomes, it had 8 *ZmAAO* on chromosome 3 with the most distribution, and 5 *ZmAAO* genes on chromosome 7, there was one *ZmAAO* on the chromosome 2, while other chromosomes contained 2 - 4 *ZmAAO* genes, which were mainly distributed in the lower part of the chromosome (Fig. 1). *ZmAAO* genes on chromosome 3 basically exist in the form of gene clusters.

Table 1. Ka/Ks analysis for the duplicated *AAO* genes.

Duplicated genes		*Ka*	*Ks*	*Ka/Ks*	Divergence time (Million years)	Duplicated type
ZmAAO6	*ZmAAO7*	0.3912	0.7358	0.5316	56.60	Tandem replication
ZmAAO9	*ZmAAO10*	0.1581	0.3168	0.4992	24.37	Tandem replication
ZmAAO34	*ZmAAO35*	0.0166	0.0429	0.3869	3.30	Tandem replication
ZmAAO12	*ZmAAO21*	0.1991	0.3701	0.5380	28.47	Segmental replication
ZmAAO12	*ZmAAO30*	0.0264	0.1290	0.2043	9.92	Segmental replication
ZmAAO19	*ZmAAO15*	0.0464	0.1507	0.3078	11.59	Segmental replication
ZmAAO28	*ZmAAO8*	0.2650	0.4900	0.5409	37.69	Segmental replication

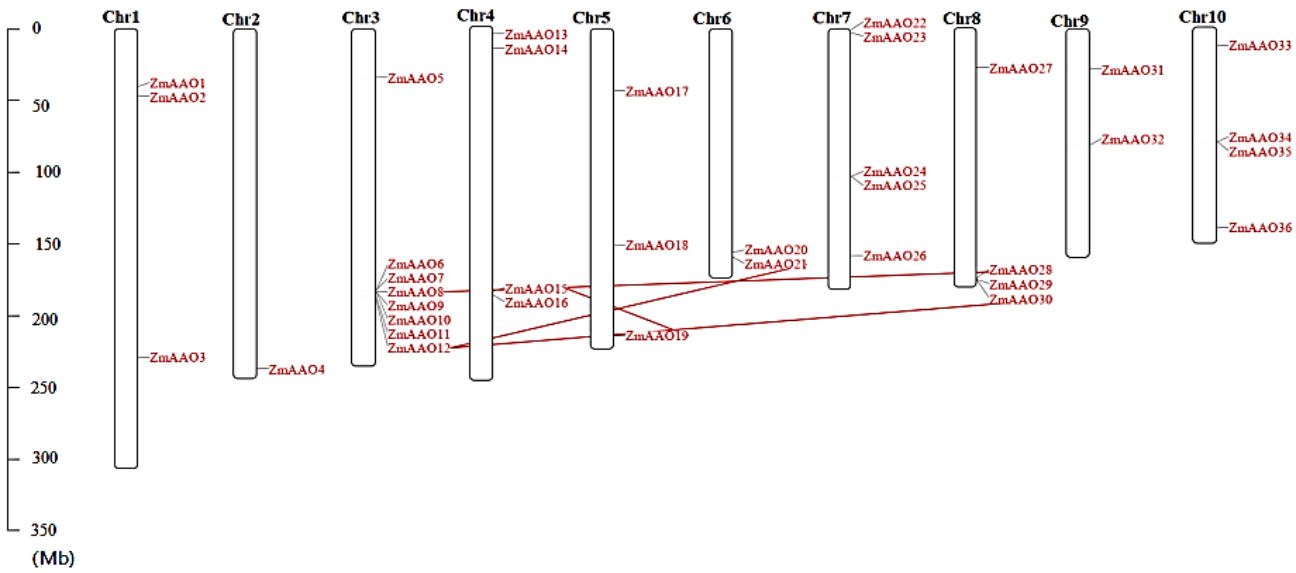

Fig. 1. Location and gene duplications of *ZmAAO* genes on maize chromosomes.
Note: The solid line indicates the genes are segmental duplication. The "Chr" at the top of each bar represent the chromosome number of maize.

Duplication and *Ka / Ks* value analysis of *ZmAAO* gene: According to the evolutionary relationship between family members and chromosomal location, a total of 7 gene pairs were found to participate in gene duplication events, except for the three gene pairs *ZmAAO6* / *ZmAAO7*, *ZmAAO9* / *ZmAAO10*, *ZmAAO34* / *ZmAAO35*, which were tandem duplication, and the other four pairs of genes were fragment duplication, indicating that the expansion of the maize *AAO* family was mainly due to fragment duplication events. The gene differentiation formed by tandem duplication has appeared in 3.3-56.60 million years, and the gene differentiation formed by fragment duplication has appeared in 9.92-37.69 million years, indicating that the gene differentiation formed by tandem duplication occurred earlier than fragment duplication. It can be seen from Table 1 that there were 7 pairs of replicators *Ka* that are lower than the synonymous substitution rate Ks, indicating that synonymous substitution has a dominant advantage, and 7 replicators have been found to have *Ka / Ks* value was less than 1, indicating that these four gene pairs have undergone strong purification selection and their functions have not undergone serious differentiation.

Structure analysis of ZmAAO protein

Analysis of protein physicochemical properties: Protein can more accurately reflect the regulation of phenotype, which was the ultimate embodiment of life activities. The results of physical and chemical analysis showed that the amino acid length of the protein was 208aa to 666aa, and the length difference was relatively large with molecular mass as 22478.62 ~ 71356.80, and the number of amino acids was proportional to the molecular weight. The isoelectric point ranged from 5.35 to 9.81, and most of them were basic proteins. It was generally believed that when the instability coefficient of a protein was greater than 40, the protein was an unstable protein. Most of the instability coefficients of the maize AAO protein in this study were less than 40, so they were mostly stable proteins. Through the analysis of total average hydrophobicity, it was found that most of the hydrophobicity of the ZmAAO protein was negative, and it was presumed that there may be more hydrophilic proteins (Table 2).

The prediction results of subcellular localization show that it has 14 *ZmAAO* genes localized in chloroplasts, 9 genes localized in vacuoles, 3 genes localized in cytoplasm, 3 genes localized in plasmodesma, 2 genes localized outside cells, 2 genes localized in mitochondrion, 1 gene localized in the nucleus, and 1 gene localized in the endoplasmic reticulum, and 1 gene localized in the peroxisome. The results show that *ZmAAO* gene has certain distribution characteristics, and it was speculated that it has a variety of functions. Through multiple mechanisms and multiple organelles to cooperate with each other to eliminate excess reactive oxygen species in the cell, thereby improving stress resistance.

Analysis of protein secondary structure: The composition of the protein determines the function of the protein, so the secondary structure of ZmAAO was analyzed using SOMPA. From the proportion, it could be seen that the random curl> extended chain structure> α-helix> β-turn. The ratio of random curl was concentrated at about 50%, and the ratio of β-turn was less than 10% (Table 3). It was speculated that the random curl structure may play a major role in the secondary structure of the protein, and the β-turn play a certain modification role. Signal peptide analysis found that all ZmAAO (except ZmAAO31) had signal peptides, which belonged to secreted protein.

Table 2. The physicochemical properties and subcellular localization of AAO protens in *Zea mays.*

Gene name	Gene ID	No. of amino acids	Molecular weight	Isoelectric point	Coefficient of instability	Grand average hydrophobicity	Subcellular localization
ZmAAO1	100193025	576	62669.76	8.65	32.87	-0.012	chlo
ZmAAO2	100279407	572	63190.88	5.93	36.04	-0.085	vacu
ZmAAO3	732784	602	66221.69	8.69	35.67	-0.229	chlo
ZmAAO4	103648173	625	68726.53	5.41	55.16	-0.185	vacu
ZmAAO5	100191779	601	65732.8	6.07	36.82	-0.138	chlo
ZmAAO6	606456	560	58774.92	5.41	37.88	0.214	vacu
ZmAAO7	103651008	569	61778.17	5.61	38.84	-0.007	extr
ZmAAO8	732847	582	63913.65	8.87	35.31	-0.144	chlo
ZmAAO9	100281551	560	61213.96	8.01	30.34	0.026	chlo
ZmAAO10	732787	585	63601.07	8.74	29.13	0.065	chlo
ZmAAO11	100282094	560	61974.95	5.77	31.97	-0.255	pero
ZmAAO12	100194388	557	62279.16	8.66	36.13	-0.248	vacu
ZmAAO13	100501262	637	69380.6	5.89	41.62	0.047	cyto
ZmAAO14	103652871	425	46390.04	9.73	47.27	-0.19	nucl
ZmAAO15	732839	666	71356.8	6.78	40.4	0.038	E.R.
ZmAAO16	103654275	571	62992.55	5.81	35.66	-0.061	extr
ZmAAO17	100281206	480	52728.58	6.71	40.24	-0.129	chlo
ZmAAO18	103628539	208	22478.62	8.95	43.96	0.103	chlo
ZmAAO19	103627746	607	65641.15	7.62	42.98	0.057	vacu
ZmAAO20	732786	587	63818.85	9.81	37.76	-0.013	mito
ZmAAO21	100285598	559	62022.69	8.72	37.95	-0.198	vacu
ZmAAO22	103631856	612	66147.08	6.06	32.79	0.017	chlo
ZmAAO23	103643803	547	58537.07	5.99	41.86	-0.026	cyto
ZmAAO24	100382137	569	62456.84	7.7	39.51	-0.222	chlo
ZmAAO25	103632652	574	63236.63	6.36	34.5	-0.19	chlo
ZmAAO26	100285637	550	60427.41	9.41	39.23	-0.174	plas
ZmAAO27	103636838	560	61246.18	7.37	40.19	-0.238	mito
ZmAAO28	100280258	584	63423.7	8.48	30.07	0.047	chlo
ZmAAO29	100502492	560	62074.33	5.66	39.35	-0.251	chlo
ZmAAO30	100285297	559	62155.83	8.67	35.29	-0.255	vacu
ZmAAO31	100383470	592	65802.51	6.01	30.97	-0.237	plas
ZmAAO32	100273169	580	63447.98	6.44	34.09	-0.136	chlo
ZmAAO33	103640860	585	63625.04	5.7	34.57	-0.094	vacu
ZmAAO34	100285611	577	64323.88	5.35	34.16	-0.386	cyto
ZmAAO35	103641288	562	62908.41	5.42	32.97	-0.352	plas
ZmAAO36	103641985	544	59782.7	9.08	37.99	-0.239	vacu

Note: Cyto: Cytoplasm; Chlo: Chloroplast; Mito: Mitochondria; Plas: Plasmodesmata; Nucl: Nucleus; Pero: Peroxisome; Vacu: Vacuoles; Extra: Extracellular; E.R.: Endoplasmic reticulum

Table 3. Secondary structure and predicted signal peptide of AAO protein in *Zea mays*.

Gene name	Alpha helix	Beta turn	Random coil	Extended strand	Signal peptide position	Cleavage site
ZmAAO1	79(13.72%)	36(6.25%)	298(51.74%)	163(28.30%)	1-28	28-29
ZmAAO2	88(15.38%)	40(6.99%)	283(49.48%)	161(28.15%)	1-27	27-28
ZmAAO3	85(14.12%)	42(6.98%)	304(50.50%)	171(28.41%)	1-22	22-23
ZmAAO4	100(16.00%)	39(6.24%)	321(51.36%)	165(26.40%)	1-23	23-24
ZmAAO5	80(13.31%)	43(7.15%)	317(52.75%)	161(26.79%)	1-26	26-27
ZmAAO6	103(18.39%)	43(7.68%)	256(45.71%)	158(28.21%)	1-22	22-23
ZmAAO7	83(14.59%)	32(5.62%)	296(52.02%)	158(27.77%)	1-21	21-22
ZmAAO8	90(15.46%)	45(7.73%)	285(48.97%)	162(27.84%)	1-38	38-39
ZmAAO9	78(13.93%)	32(5.71%)	293(52.32%)	157(28.04%)	1-26	26-27
ZmAAO10	87(14.87%)	36(6.15%)	305(52.14%)	157(26.84%)	1-29	29-30
ZmAAO11	69(12.32%)	37(6.61%)	302(53.93%)	152(27.14%)	1-34	34-35
ZmAAO12	79(14.18%)	35(6.28%)	278(49.91%)	165(29.62%)	1-28	28-29
ZmAAO13	103(16.17%)	45(7.06%)	311(48.82%)	178(27.94%)	1-41	-
ZmAAO14	58(13.65%)	28(6.59%)	229(53.88%)	110(25.88%)	1-29	-
ZmAAO15	136(20.42%)	38(5.71%)	326(48.95%)	166(24.92%)	1-53	-
ZmAAO16	89(15.59%)	35(6.13%)	289(50.61%)	158(27.67%)	1-27	27-28
ZmAAO17	59 (12.29%)	31(6.46%)	238(49.58%)	152(31.67%)	1-29	29-30
ZmAAO18	36(17.31%)	16(7.69%)	87(41.83%)	69(33.17%)	1-18	-
ZmAAO19	90(14.83%)	38(6.26%)	315(51.89%)	164(27.02%)	1-32	32-33
ZmAAO20	86(14.65%)	40(6.81%)	299(50.94%)	162(27.60%)	1-32	32-33
ZmAAO21	92(16.46%0	40(7.16%)	267(47.76%)	160(28.62%)	1-27	27-28
ZmAAO22	125(20.42%)	38(6.21%)	301(49.18%)	148(24.18%)	1-29	-
ZmAAO23	82(14.99%)	31(5.67%)	272(49.73%)	162(29.62%)	1-23	23-24
ZmAAO24	64(11.25%)	33(5.80%)	311(54.66%)	161(28.30%)	1-18	18-19
ZmAAO25	72(12.54%)	40(6.97%)	297(51.74%)	165(28.75%)	1-27	27-28
ZmAAO26	81(14.73%)	42(7.64%)	258(46.91%)	169(30.73%)	1-22	22-23
ZmAAO27	76(13.57%)	40(7.14%)	296(52.86%)	148(26.43%)	1-53	-
ZmAAO28	80(13.70%)	43(7.36%)	297(50.86%)	164(28.08%)	1-31	31-32
ZmAAO29	72(12.86%)	40(7.14%)	291(51.96%)	157(28.04%)	1-42	42-43
ZmAAO30	85(15.21%)	38(6.80%)	272(48.66%)	164(29.34%)	1-30	30-31
ZmAAO31	100(16.89%)	36(6.08%)	283(47.80%)	173(29.22%)	1-21	21-22
ZmAAO32	81(13.97%)	37(6.38%)	307(52.93%)	155(26.72%)	1-24	24-25
ZmAAO33	82(14.02%)	37(6.32%)	308(52.65%)	158(27.01%)	1-31	31-32
ZmAAO34	89(15.42%)	35(6.07%)	284(49.22%)	169(29.29%)	-	-
ZmAAO35	86(15.30%)	34(6.05%)	280(49.82%)	162(28.83%)	1-16	16-17
ZmAAO36	72(13.24%)	33(6.07%)	267(49.08%)	172(31.62%)	1-20	20-21

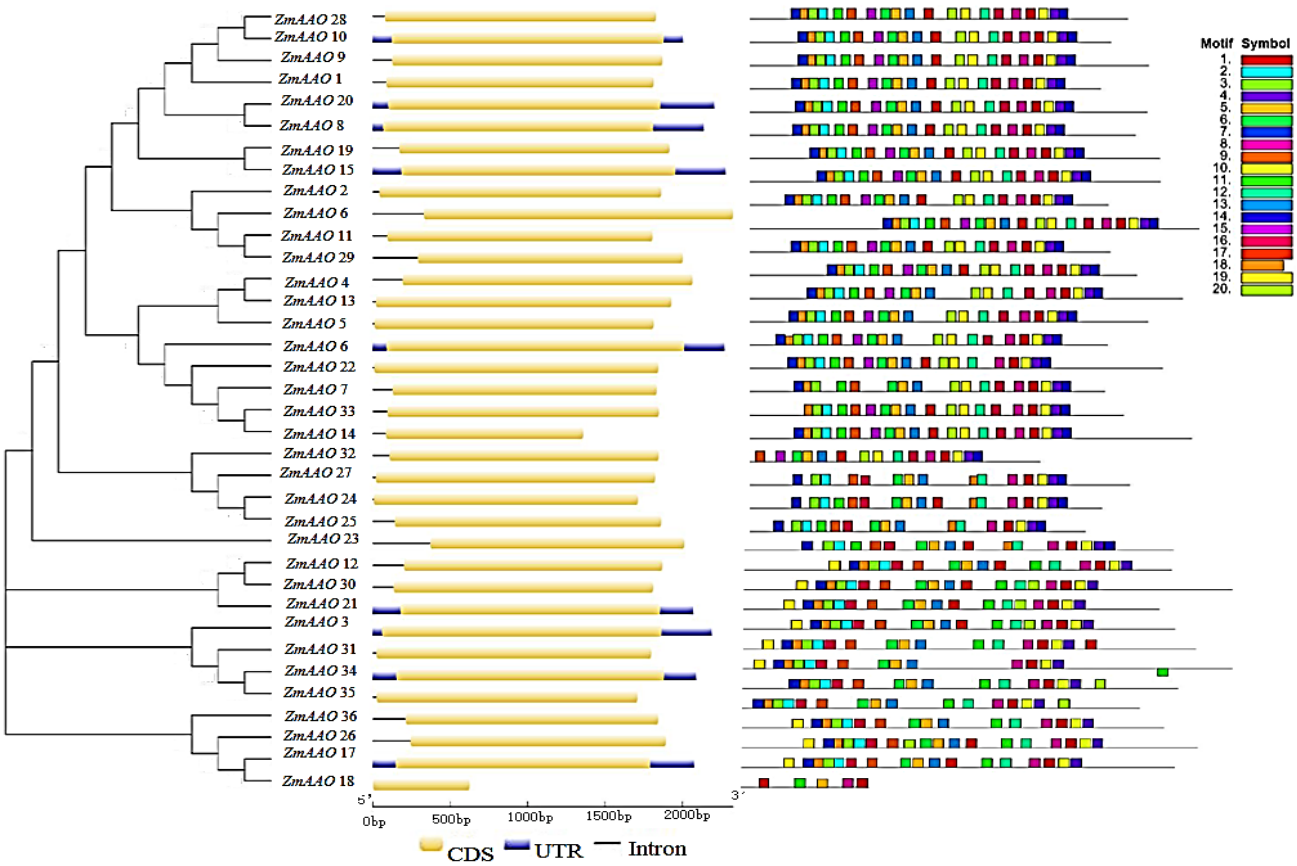

Fig. 2. Gene structure and Conserved motifs analysis of *AAO* genes in maize.
Note: The yellow boxes represent exons; Line represent introns; The blue boxes represent UTR. Motifs of the ZmAAO proteins were identified using the online MEME program. Different coloured boxes represent different motifs.

Analysis on structure of *ZmAAO* genes and conserved motifs of *ZmAAO* proteins: Gene Structure Display Server online software was used to draw the structure distribution diagram. There were some differences in the number and length of introns and UTRs of 36 *AAO* genes in maize, but the changes were not significant (Fig. 2). Among them, *ZmAAO2*, *ZmAAO5*, *ZmAAO6*, *ZmAAO8*, *ZmAAO16*, *ZmAAO28*, *ZmAAO29*, *ZmAAO31*, and *ZmAAO35* all contain 2 UTRs without contained introns, while *ZmAAO36* has only coding sequence (CDS).

The MEME online tool was used to the conserved motifs analysis of maize AAO protein sequences, the results showed that maize AAO-like proteins have 20 conserved motifs and have large length changes, which indicates that the members of the AAO family gene had relatively higher conservatism. Further analysis of the distribution of these conserved motifs in the maize AAO protein (Fig. 2) was found that most AAOs contain 20 conserved motifs, and there were also a few genes with conserved motifs missing, of which the ZmAAO36 conserved motif were left 5 conserved motifs with the most severe deletions.

Phylogenetic analysis of ZmAAO protein: After searching in the NCBI database and testing with Smart and CDD programs, 118 AAO homologous sequences were finally identified, of which 42 AAOs came from *Sorghum bicolor*, 41 AAOs came from *Oryza sativa*, and 35 AAOs came from *Arabidopsis thaliana*. In order to further explore the evolutionary relationship of the AAO family, the MEGA-X software was used to construct a phylogenetic tree of the AAO family (Fig. 3) in this study. As it could be seen from Fig. 3, these AAOs were aggregated into 4 groups, and the number of AAOs in group D was the smallest, containing only 7 AAOs from 3 species (3 AAOs from *Oryza sativa*, 2 AAOs from maize, 2 AAOs from *Sorghum bicolor*), and the remaining groups contained AAOs of 4 plant species. Group C contained the most AAOs (total in 78 AAOs, among which 18 AAOs from *Oryza sativa*, 16 AAOs from maize, 16 AAOs from *Sorghum bicolor* and 28 AAOs from *Arabidopsis thaliana*), and group B contained 43 AAOs (*Oryza sativa*: 15; maize: 11; *Sorghum bicolor*: 14 and *Arabidopsis thaliana*: 3); group A contained 26 AAOs (*Oryza sativa*: 5; maize: 7; *Sorghum bicolor*: 10; *Arabidopsis thaliana*: 4).

A total of 126 AAOs were found to be homologous, accounting for 81.82% of the total number of genes in this study. There were 49 pairs of orthologous AAOs among the species, including 12 pairs from maize and *Oryza sativa*, 10 pairs from maize and *Sorghum biocolor*, 3 pairs from maize and *Arabidopsis thaliana*, 12 pairs from *Oryza sativa*, *Sorghum bicolor*, 6 pairs from *Oryza sativa* and *Arabidopsis thaliana*, 6 pairs from *Sorghum bicolor* and *Arabidopsis thaliana*. There were more orthologous AAOs came from maize, *Oryza sativa* and *Sorghum bicolor*, and these species was closer in genetic relationship. The orthologous AAO of maize and *Arabidopsisis thaliana* was the least and their genetic relationship is far; there were 14 pairs of paralogous AAOs in the species, 6 pairs of which from *Arabidopsis thaliana*, 3 pairs from maize, 3 pairs from *Sorghum bicolor*, and 2 pairs from *Oryza sativa*.

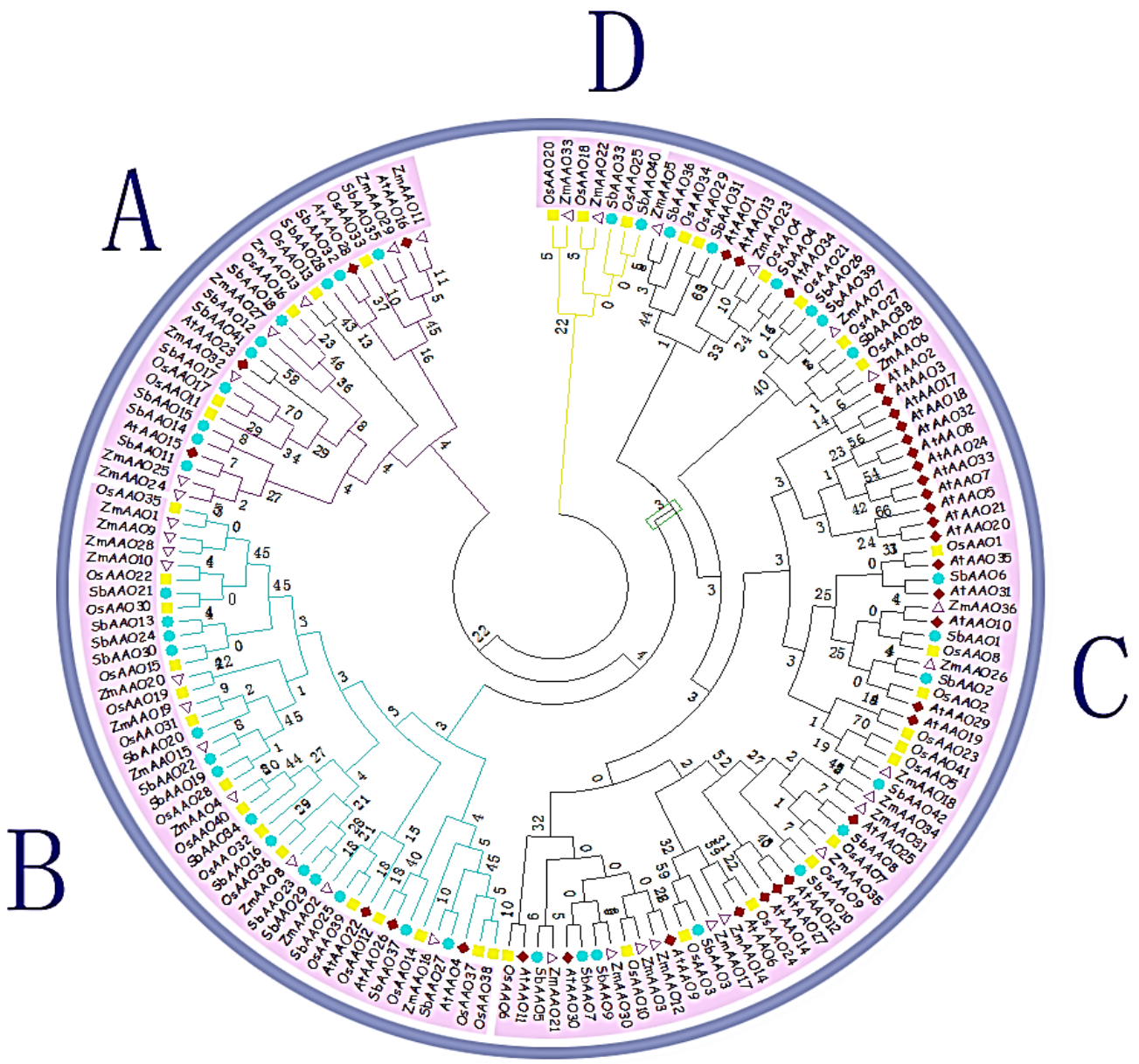

Fig. 3. Phylogenetic relationship of AAO proteins from maize, sorghum, Arabidopsis and rice.

Expression analysis of *ZmAAO* genes: In this study, tissue expression data of the *ZmAAO* gene family were obtained from the NCBI SRA database and normalized. From its expression profile (Fig. 4), the *ZmAAO6*, *ZmAAO17* and *ZmAAO34* genes were all highly expressed in different tissues, while the *ZmAAO14*, *ZmAAO18* and *ZmAAO25* genes were lower expressed in different tissues (the expression value was negative); *ZmAAO32* had higher expression levels in the roots, stalks, silks, ears, and tassels and with lower expression levels in leaves; *ZmAAO26* had higher expression levels in roots, stalks, leaves, silks, and ears and with low expression level in tassels. It was the most highly expressed genes in the roots (reached to 18 genes), followed by the stalks (13 genes), followed by tassels (9 genes), leaves (7 genes), ears (7 genes), and silks (6 genes).

The expression of 36 *ZmAAO* genes was significantly different under high temperature stress (2h, 48h). The expression levels of most genes decreased after high temperature stress, and the expression levels of some genes decreased first and then increased, such as *ZmAAO6*, *ZmAAO10*, *ZmAAO11*, *ZmAAO15*, *ZmAAO16*, *ZmAAO29* and *ZmAAO35* genes in ears; *ZmAAO34* and *ZmAAO35* genes in silks; *ZmAAO12*, *ZmAAO15*, *ZmAAO21*, *ZmAAO30*, *ZmAAO32* and *ZmAAO36* in tassels; *ZmAAO23*, *ZmAAO26*, *ZmAAO31* and *ZmAAO32* in stalks; *ZmAAO1*, *ZmAAO8*, *ZmAAO19*, *ZmAAO20*, *ZmAAO21*, *ZmAAO24*, *ZmAAO28*, *ZmAAO31* and *ZmAAO34* in the roots; *ZmAAO1*, *ZmAAO8*, *ZmAAO9*, *ZmAAO11*, *ZmAAO20*, *ZmAAO22*, *ZmAAO26*, *ZmAAO28*, *ZmAAO29* and *ZmAAO34* in the leaves; the expression of some genes increased first, and then decreased, such as *ZmAAO6*, *ZmAAO10*, *ZmAAO11*, *ZmAAO20*, *ZmAAO22* and *ZmAAO29* in the roots; *ZmAAO17*, *ZmAAO31*, *ZmAAO34* and *ZmAAO35* in the stalks; *ZmAAO6*, *ZmAAO15* and *ZmAAO19* in the tassels; *ZmAAO17* and *ZmAAO23* in the silks; *ZmAAO17* and *ZmAAO35* in the ears; the expression level of a few genes is gradually increasing, such as *ZmAAO6*, *ZmAAO17* and *ZmAAO31* in leaves; and *ZmAAO15* in roots; *ZmAAO6* in silks; *ZmAAO33* in tassels.

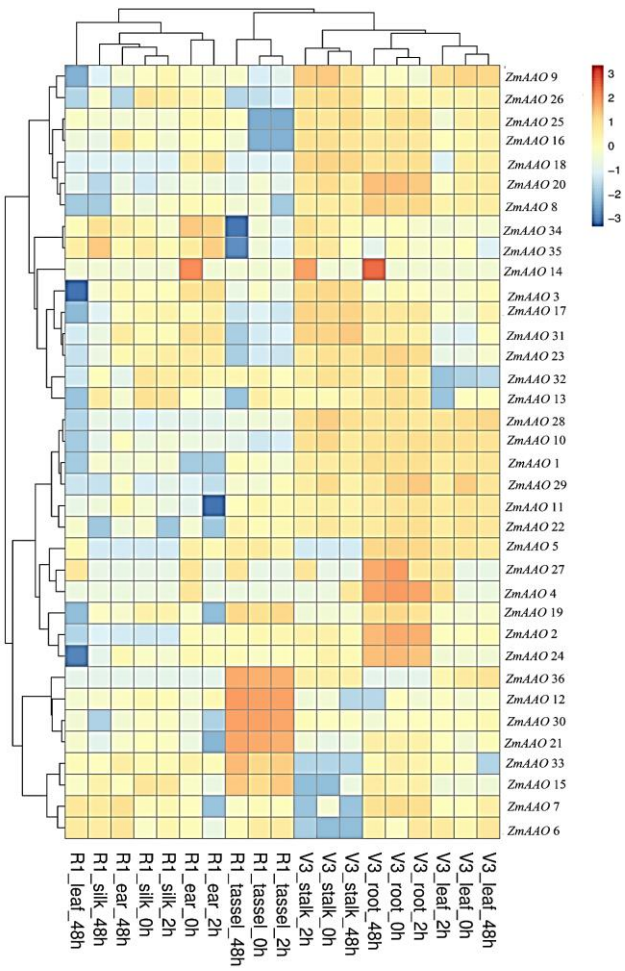

Fig. 4. Heat map of transcriptome analysis of *ZmAAO* gene family in three leaf stage under heat stress.
Note: V3: Three leaf stage; R1: Loose powder period; heat stress time: 0h, 2h, 48h.

Discussion

AAO is a blue copper protein that uses ASA as an electron donor and to oxidizes it into water (Tullio *et al.*, 2007). It plays an important regulatory role in plant growth and development (Pignocchi *et al.*, 2003), flowering sequential (Yamamoto *et al.*, 2005), stress response of adversity (Sanmartin *et al.*, 2003; Sanmartin *et al.*, 2007; Fotopoulos *et al.*, 2006) and in the various metabolic pathway (Pignocchi *et al.*, 2003) .

The amino acid sequence encoded by ZmAAO contains three domains, Cu-oxidase_3, Cu-oxidase and Cu-oxidase_2, which have the same conserved domains as other species of AAO (Wang *et al.*, 2013; Qiao *et al.*, 2016). Based on this characteristic, 118 AAO homologous protein sequences was identified from the NCBI database in this study. The phylogenetic tree was preliminarily divided into 4 subfamilies, among which group D contains only maize, sorghum and rice, indicating that maize *AAO* gene was closely related to *Sorghum bicolor* and *Oryza sativa*. These predictive genes could also be used to improve the varieties of these major food crops in the future. In addition, it was found that *ZmAAO* genes were localized in different organelles. It was speculated that different organelles may cooperate with each other through multiple mechanisms to remove ROS, achieved antioxidant functions, and maintained the dynamic balance of free radicals in the body.

The secondary structure of protein is a stable structure formed by different amino acid residues form regular repeats of the peptide chain by means of hydrogen bonds formed between C = O and N-H (Cao *et al.*, 2010), which were mainly divided into α-helix,β- turn and irregular curl. These are the basis for the spatial structures of protein. Different amino acid residues tend to form different secondary structural elements (Li *et al.*, 2012). Predicting and analyzing protein structure is important for understanding the relationship between protein structure and diverse functions, and protein interactions. This study found that the highest proportion of irregular curls in the 36 ZmAAO amino acid sequences may be likely to be closely related to the function of ZmAAO.

Previous studies have found that the expression and activity of *AAO* in *Matrimony vine* (Qiao *et al.*, 2016) and *Oryza sativa* (Ke *et al.*, 2019) showed a positive correlation, with the highest expression and activity in flowers, the second in fruits, and with the lowest in mature leaves. Over-expression of the *AAO* gene in tobacco will increase the growth rate (Pignocchi *et al.*, 2003), bloom early, reduce the number of flower buds, and reduce the seed setting rate (Yamamoto *et al.*, 2005). However, this study showed that the expression of *AAO* in roots, stalks, and leaves was higher level, and that of silks, tassels, and ears was lower, indicating that the *AAO* gene is expressed differently in different species. The high expression of *ZmAAO* gene may lead to increased AAO enzyme activity, thereby promoting growth and development of maize.

The plants are facing many challenges due to climate change. The Global Climate Change Analysis (GCCA) predicts that the average temperature will rise by 1-3.7℃ at the end of the 21st century (Team *et al.*, 2014). Researchers have adopted different technologies to enhance the potential of plants to respond to climate change, including high temperature stress (Ismail *et al*., 2019); the plant itself also reduces damage through high temperature stress through some means such as increased protective enzyme activity (Ren *et al.*, 2019). In this study, after the maize was subjected to high temperature stress, the *AAO* genes increased in the tissues, such as *ZmAAO6*, *ZmAAO17* and *ZmAAO31* in the leaves, *ZmAAO15* in the roots, *ZmAAO6* in the silks, and *ZmAAO33* in the tassels, which indicated that these *AAO* genes might play a protective role in maize tissues under high temperature stress.

In conclusion, this study analyzes the structure and function of the *ZmAAO* gene family through bioinformatics, which provides a theoretical basis for later study of the biological function in the gene family by molecular biology methods, and has the reference significance for the cultivation of maize varieties with high temperature tolerance.

Conclusion

36 *ZmAAO* genes were unevenly distributed on 10 chromosomes. The genes differentiation was dominated by fragment replication, and the differentiation time was about 3.3-25.75 million years. ZmAAO proteins were mostly basic

proteins; The protein secondary structure is mainly composed of irregular curls; The analysis of gene structure and conserved motifs showed that there was no significant difference in the number of introns in *ZmAAO* genes, and the amino acid sequence was highly conserved. The phylogenetic trees can be divided into 4 subfamilies, in which maize, rice and sorghum has a large number of orthologous genes and close genetic relationships. The expression pattern analysis found that the expression level of *ZmAAO* was different in different tissues, which indicates that it functions differently. After high temperature stress, the expression level of *ZmAAO* gene was significantly different in different tissues, indicating that some *ZmAAO* genes respond to high temperature stress.

Acknowledgments

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