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Identification of *Populus tomentosa* OSCA Gene Family and Its Expression Analysis under Salt Stress

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Abstract *OSCA* (Hyperosmdality-gate calcium-permeable channels) is a calcium-permeable cation channel protein. By participating in sensing changes in extracellular osmotic potential, it induces an increase in Ca^{2+} concentration, regulates osmotic potential, and plays an important role in hypertonic stress induction. However, there are few studies on the *OSCA* genes of poplar. In this study, 16 *OSCA* genes sequences were identified from *Populus tomentosa* genome, and renamed them according to their distribution on the chromosomes. Based on phylogenetic tree analysis, the *PtOSCAs* genes were divided into 4 groups, and the *OSCA* genes in each group had similar gene structures. Two repetitive events were identified between poplar OSCA family genes, one tandem repetitive event involving 3 genes and a fragment repetitive event involving 2 genes, and the Ka/Ks value of each pair of genes was below 1. Therefore, it can be seen that the OSCA family genes of poplar have a purification effect during evolution. By analyzing the expression of *PtOSCAs* gene under low and high concentration salt stress, it is found that there are the following three types: Type I, where the expression level increases under both low and high concentration salt stress; Type II, at low and high concentration salt stress, the expression level decreased under high concentration salt stress; Type III, the expression level showed an upward trend under low concentration salt stress, but the expression level showed a downward trend under high concentration salt stress. This study provides a reference for the functional analysis of *PtOSCAs* gene and the genetic improvement of poplar.

Keywords *Populus tomentosa*; *OSCA*; Salt stress; Expression characteristics

There are many stress effects (such as salt-alkali, high temperature and drought) in nature, which will adversely affect the growth and development of plants, and even lead to plant death. In nature, plants have formed mature defense mechanisms during long-term evolution to adapt to complex environmental changes (Fu et al., 2019; Maity et al., 2019). As the second messenger, Ca^{2+} plays an extremely important role in plant cell signal transduction. After the stimulation of the external environment on plants, the intracellular free Ca^{2+} (Intracellular Ca^{2+}) concentration in the cytoplasm of plants can be effectively regulated by the transport system. For example, the production of corresponding physiological reactions can be promoted through Ca^{2+} channels (Batistic and Kudla, 2012). Both abiotic stress (Zhu et al., 2002; Bartels and Sunkar, 2005) and biotic stress (Lecourieus et al., 2006) can induce plant resistance to stress by activating Ca^{2+} pathways.

Ca^{2+} concentration is directly related to the calcium ion pathway. Therefore, the study of Ca^{2+} pathway is of great significance to clarify plant response to stress and regulate plant stress resistance. In early studies, it has been found that some proteins are widely used, such as cyclic nucleotide-gated ion channel (CNGC), glutamate receptor family (GLR), and complementary active protein (MCA) (Wang et al., 2017; Cao et al., 2020). In 2014, the OSCA gene family was first studied in Arabidopsis genome (Yuan et al., 2014) and 15 members were found in

this family, all of which have DUF221 domain related to plant stress response (Yuan et al., 2014). 11 OSCA gene family members were identified in rice (Li et al., 2015), 21 OSCA gene family members were identified in soybean (Li et al., 2017). From the above, we know that the OSCA gene family is likely to widely exist in other plants.

In rice, 10 differentially expressed *OsOSCA* genes were induced by osmotic stress (ABA, NaCl, PEG), among which *OsOSCA3.1* was identified to be involved in early drought response *OsERD4* gene (Rai et al., 2012; Yuan et al., 2014). *OsOSCAI.4* in rice, *AtOSCAI.8* in *Arabidopsis thaliana* and *AtOSCA1.4* in wheat all belong to the same family, which are directly affected by related plant traits such as wheat yield, grain number per spike, apical sterility (Lü, 2015). There are 13 *GmOSCAs* genes in soybean, which are directly affected by salt-alkali stress and drought response (Li et al., 2017). Therefore, it can be found that this gene is mainly related to abiotic stress, but the correlation between *OSCA* gene and abiotic stress in poplar has not been reported.

Poplar has a long history of cultivation and a wide range of uses. It is an economic tree species for ecological protection forests and industrial timber (Tun et al., 2018) and plays an extremely important role worldwide. With the aggravation of soil salinization, salt stress has become an important factor restricting the growth of trees (Du et al., 2012). Therefore, the study of gene function related to salt tolerance has attracted more and more attention. However, up to now, there is no in-depth systematic study on the identification and expression pattern of poplar OSCA gene family. To realize the research on this issue, the OSCA gene family members of poplar genome were obtained through bioinformatics identification in this study, and then the gene structure, evolution rate and salt stress of related members were analyzed. This study provides a reference for the further study on the biological function of poplar *OSCA* gene and the molecular mechanism of response to stress environment.

1 Results and Analysis

1.1 Identification and analysis of OSCA gene family in poplar

The identified Arabidopsis OSCA protein sequence was used as the query sequence, and BLASTP search and screening were performed on the subjects in this study. The redundant sequences in the gene sequences were removed manually, and the gene sequences were verified by InterProScan and SMART databases. After that, 16 *OSCA* gene sequences were obtained. With the application of biological information method, the analysis of related parameters of family genes is carried out, including basic properties such as molecular weight, sequence length and subcellular localization, and the renaming of 16 genes is completed with the application of position on gene chromosomes (Table 1). The results showed that 16 *PtOSCAs* genes were located on chromosomes. The length of *PtOSCAs* gene sequence varies greatly, among which *PtOSCA13* gene sequence is the shortest with the length of 1 482 bp, while *PtOSCA11* of 2 505 bp. There was no significant difference in the length between CDs sequence and protein sequence, with an average of 2 069 bp and 737 aa. The results showed that the molecular weight range of *PtOSCAs* gene was concentrated in 56.32~94.89 kD, and the average molecular weight was 83.97 kD. The subcellular localization prediction results showed that 16 *PtOSCAs* genes were localized on the plasma membrane.

1.2 Phylogenetic analysis and classification of *OSCA* gene family in poplar

In the research, the similar regions and conserved sites between *PtOSCAs* genes were studied to clarify the evolutionary relationship of species. In this study, the phylogenetic tree was constructed based on the multiple alignment of 16 PtOSCAs protein sequences and 15 AtOSCAs protein sequences, and the corresponding analysis structure. Combined with the results of phylogenetic analysis, the 31 OSCAs protein sequences were divided into 4 groups (Figure 1).

Table 1 Sequence characteristics of *OSCA* gene family in *P. trichocarpa*

Name	Gene ID	Chr	Genomic location	CDs	Proteins			Subcellular location
					Size	Molecular weight	pI	
PtOSCA1	POPTR_001G133800v3	1	10755520:10762863	2418	805	92.59	9.82	Plasma-membrane
PtOSCA2	POPTR_001G358300v3	1	36770547:36775147	1818	605	67.89	9.83	Plasma-membrane
PtOSCA3	POPTR_002G113800v3	2	8517988:8525919	2211	736	83.04	8.83	Plasma-membrane
PtOSCA4	POPTR_002G226800v3	2	21609893:21615367	2292	763	87.17	9.03	Plasma-membrane
PtOSCA5	POPTR_003G099800v3	3	12577160:12583882	2427	808	93.15	9.81	Plasma-membrane
PtOSCA6	POPTR_003G200900v3	3	20318923:20324092	2181	726	82.34	8.03	Plasma-membrane
PtOSCA7	POPTR_004G005900v3	4	371221:377923	2325	774	88.11	9.66	Plasma-membrane
PtOSCA8	POPTR_004G006000v3	4	380346:386486	2091	696	79.36	9.01	Plasma-membrane
PtOSCA9	POPTR_004G006100v3	4	393580:398299	2328	775	88.50	8.86	Plasma-membrane
PtOSCA10	POPTR_004G115800v3	4	10681402:10691519	2151	716	81.65	8.32	Plasma-membrane
PtOSCA11	POPTR_005G108600v3	5	8332404:8335565	2505	834	94.89	6.7	Plasma-membrane
PtOSCA12	POPTR_007G063700v3	7	8026602:8029479	2439	812	92.51	6.61	Plasma-membrane
PtOSCA13	POPTR_008G091200v3	8	5713772:5722591	1482	493	56.32	8.87	Plasma-membrane
PtOSCA14	POPTR_010G164100v3	10	16833961:16843326	2139	712	80.46	8.66	Plasma-membrane
PtOSCA15	POPTR_011G009900v3	11	753089:761429	2319	772	87.72	9.34	Plasma-membrane
PtOSCA16	POPTR_014G156100v3	14	12167104:12176916	2307	768	87.75	9.34	Plasma-membrane

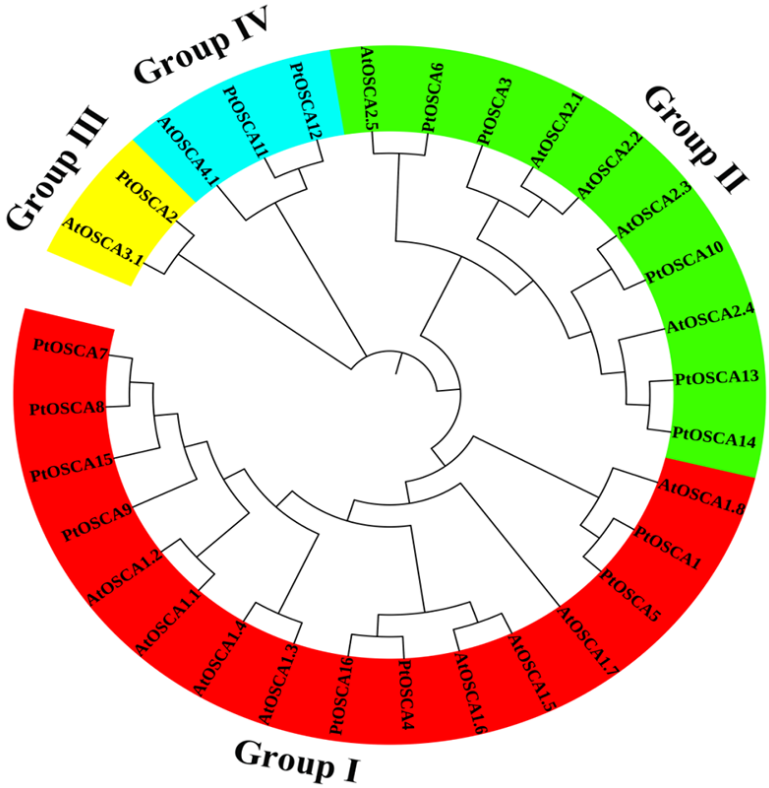

Figure 1 Phylogenetic tree of OSCA family members of *poplar* and *Arabidopsis*

1.3 Structure and motif analysis of members of OSCA gene family in poplar

To study the conservation and evolutionary relationship of *OSCA* genes between different groups, the multiple alignment of exons and introns distribution among OSCA family members was carried out in this study (Figure 2). The results of gene structure analysis showed that if the number of introns in the same group was close, there was a big difference no longer in the same group. For example, the number of introns in GroupIII and GroupI is 5 and

10, respectively. In the process of this experiment, the study of exon-intron composition mode showed that if in the same group is closer, if not, there is a big difference. For example, in the investigation, it has been found that the intron sequence of GroupI was slightly longer than other sequences, and in this case, the corresponding genome sequence was also longer, showing that all of them were more than 9 200 bp. While the number of introns in GroupIV was the lowest in the sequence, and the natural sequence was the shortest, all below 3 200 bp. The above analysis of the conservatism of the gene structure among the same group of genes is completely consistent with their evolutionary relationship.

To analyze the conservatism of poplar OSCA family, the analysis of conservative motif was realized based on MEME software. 10 conserved motifs were found in PtOSCAs protein, and the length range of each conserved motif was 21~100 amino acids. Each *PtOSCAs* gene contains 3~10 conserved motifs and contains motif 1 (Figure 2). Except for GroupIV, the other 3 Group protein sequences were highly conserved, and the conservative motif composition patterns of GroupI and GroupIII proteins were close to each other. The protein sequences in GroupI were also highly conserved. However, the functional domains of Lateexocytosis at N-terminal were composed of motifs 1, 4, 5, 7, 8, 9, 15 and 16, while those in GroupII and GroupIII were composed of motifs 3, 6, 10, 13, 14 and 2, indicating that they were different in evolution and function.

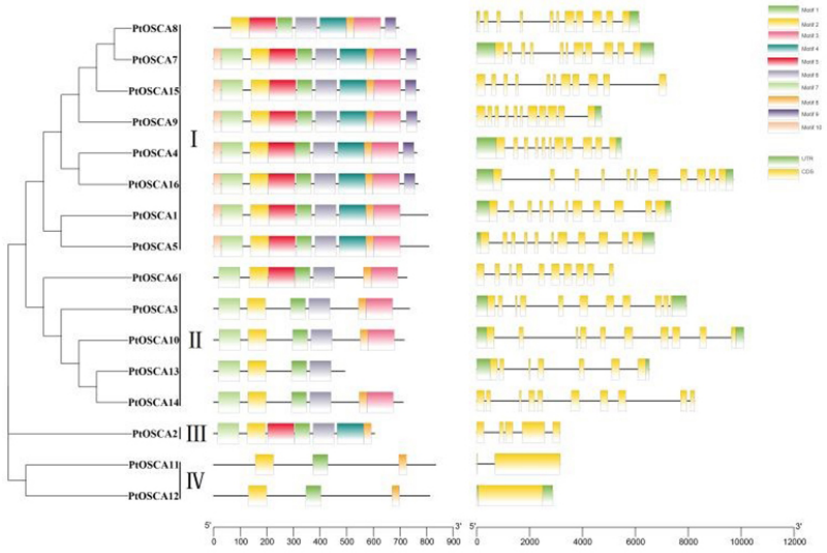

Figure 2 *Poplar OSCA* gene structure and conserved motifs

1.4 Chromosome localization and doubling event analysis of OSCA gene family in poplar

The results showed that all *PtOSCA* genes were located on chromosomes and were not evenly distributed on 10 chromosomes. Among them, *PtOSCAs* genes on chromosome 4 were the most, with 3 genes, while *PtOSCAs* genes on chromosomes 5, 7, 8, 10, 11 and 14 were the least, with only 1 gene, respectively (Figure 3).

Tandem repeats and fragment repeats play a role in the formation of gene families, and help to promote genome evolution. Therefore, in this study, we analyzed the doubling events of *PtOSCAs* gene. In poplar OSCA gene family, there are only one group with a total of 3 genes (*PtOSCA7*, *PtOSCA8* and *PtOSCA9*) identified as tandem repeat genes, located on chromosome 4. In addition, one fragment repeat event of the two genes was also identified (Figure 4). To further analyze the evolutionary pressure of *PtOSCAs* gene, the Ka/Ks values of tandem repeat and fragment repeat genes were calculated and analyzed. It was found that the Ka/Ks values of the determined genes were all below 1, indicating that the purification selection had an impact on the evolutionary process of OSCA gene family in poplar. Through the above research, we have a further understanding on the expansion mode of *OSCA* gene in poplar family.

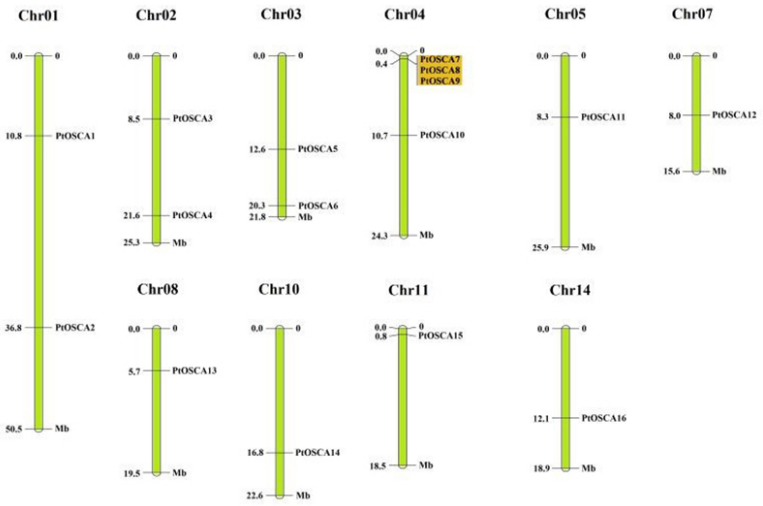

Figure 3 Chromosome mapping of members of *OSCA* gene family in *Poplar*

Note: The yellow-marked in the figure are the identified tandem repeat genes

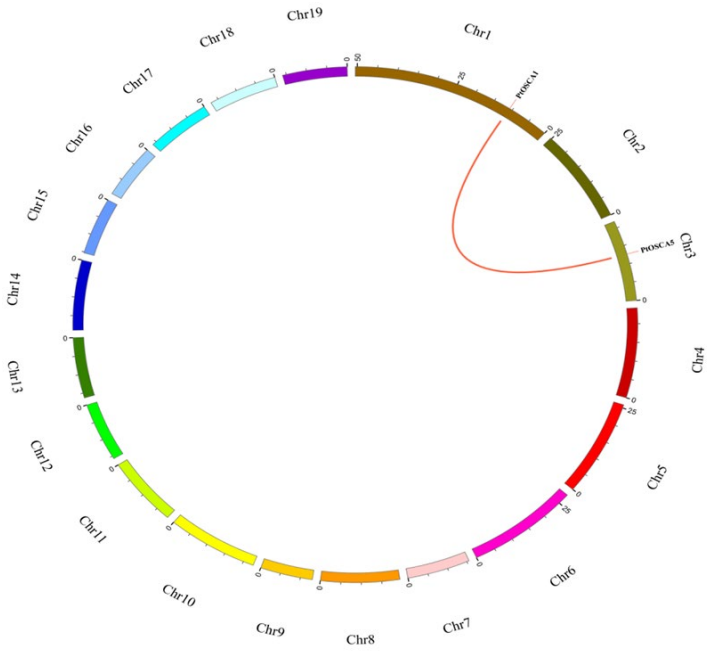

Figure 4 The synteny analysis of *OSCA* gene family in *Poplar*

Note: The circle in the figure represents the 19 chromosomes of poplar with color difference, and the red connecting gene is the fragment doubling event

1.5 Expression pattern and analysis of OSCA gene family in poplar under salt stress

According to the transcriptome data of leaf tissue of *Populus×euramericana cv.'74/76'* under different concentrations of salt stress, the expression of OSCA gene family members in poplar under salt stress was realized. The results showed that the expression of 16 *OSCAs* genes changed significantly (Figure 5), indicating that these genes were involved in the response process of poplar to salt stress. By analyzing the expression of *PtOSCAs* gene under low and high concentration salt stress, it is found that there are the following three types: Type I, where the expression level increases under both low and high concentration salt stress; Type II, at low and high concentration salt stress, the expression level decreased under high concentration salt stress; Type III, the expression level showed an upward trend under low concentration salt stress, but the expression level showed a downward trend under high concentration salt stress (Figure 5).

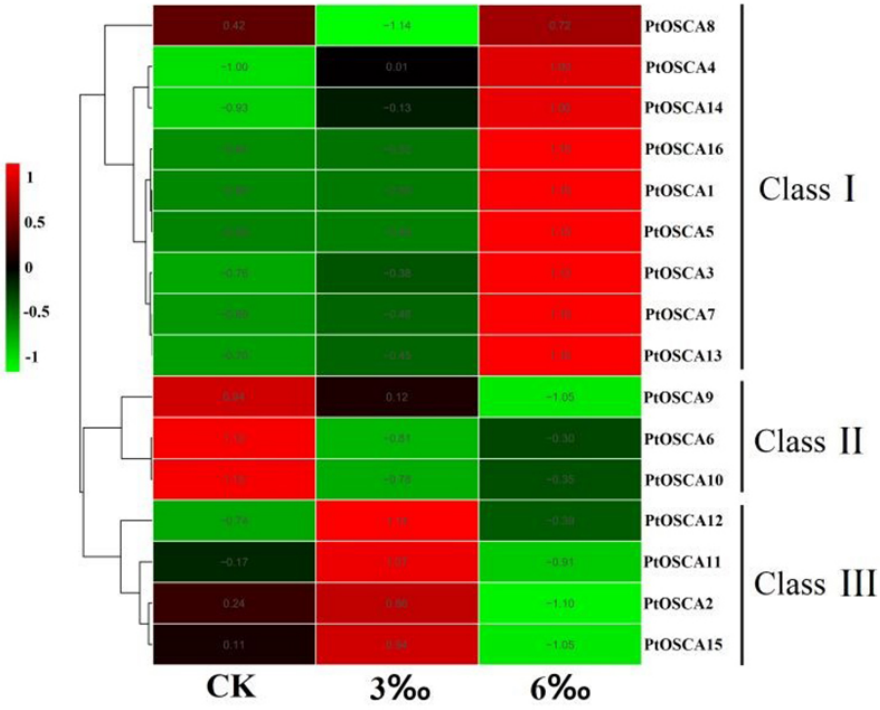

Figure 5 Heatmap of the expression of each member of the poplar OSCA gene family under salt stress

2 Discussion

The research on the OSCA gene family first appeared in *Arabidopsis thaliana* in 2014. Under the analysis of Ca^{2+} indicators and other experiments, the result of *OSCA* analysis showed that it was a Ca^{2+} receptor with strong hyperosmosis and played an important role in the molecular mechanism of drought (Li et al., 2017). At the same time, the identification of C4 crops such as sorghum and maize were carried out in diploid model plants such as rice and Arabidopsis. The results showed that *TaOSCA1.4* gene in hexaploid wheat (Lü, 2015), but so far there are few research on *OSCA* gene in poplar. In this study, a total of 16 *OSCA* gene sequences in poplar genome were found, which were more than those in Arabidopsis and rice, but less than those in soybean (Tun et al., 2018). Based on the phylogenetic analysis with Arabidopsis, 16 *PtOSCAs* were divided into four groups, each of which included OSCA family members from poplar and Arabidopsis. PGDD analysis showed that there was duplication in poplar *OSCA* gene, two genes existed at the same time called genome duplication, and all of which were in the same group. From this point, we know that genome duplication was highly dependent on gene expansion of poplar family. It can be found that there is a certain selection pressure in the evolution of poplar OSCA gene family under purification through the replication gene Ka/Ks value below 1. The above analysis showed that the poplar OSCA gene family structure is conservative.

Based on the above analysis of the evolutionary relationship, gene structure and protein structure of poplar, it is found that poplar OSCA family is highly conserved. In the study, Yuan et al. (2014) carried out a systematic classification of *Arabidopsis thaliana*, and then analyzed the evolution of maize, rice, poplar, Chlamydomonas and so on, and found that OSCAS was also divided into 4 Clade, which is consistent with the results of this study. Based on the analysis of gene results, it was found that there was a high similarity in the exon-intron structure pattern of poplar OSCA gene family if they were in the same group. Based on the protein structure analysis, the Caleium-de-pendent (DUF221) domain was found in the OSCA gene family, which was regarded as the DUF221 gene family in the research. And the protein was named hyperosmdality-gate calcium-permeable channels (Hou et al., 2014). However, in the early analysis, it was also named as early-responsive to dehydration stress proteins (ERDs) (Kiyosue et al., 1994) and trans-membrane channel-like proteins (TMC) (Chatzigeorgiou et al., 2013). The above situation is mainly due to the presence of high salt stimulation in animals. In the study, it can be seen as

sodium ion sensing proteins (TMCs) and chloride channel proteins (Caccs) based on the effect of CSCs and calcium activation are all the same superfamily (Hou et al., 2014). Based on the protein conserved domain, it was found that the number of OSCA gene sequences of poplar was 16, only Group IV was less conserved, the protein sequences of the other three 3 Groups were very conservative, Group I group was the first, and the composition patterns of the remaining two sequences were similar.

Gene doubling events are retroposition, tandem replication, fragment replication, etc., especially in gene family expansion. In 16 *PtOSCAs* genes unevenly distributed on 10 chromosomes, tandem replication and fragment replication events were identified. Through transcriptome data analysis, we found that the expression patterns of each pair of genes under salt stress are not the same, and the replication genes may change in function during evolution, which often exists in the multi-gene families of eukaryotes. According to the transcriptome data, the *PtOSCAs* gene expression patterns under different salt concentrations were obtained, and the gene expression levels were collected. *PtOSCAs* genes were divided into 3 categories during the study, and the gene expression patterns were consistent. In the process of gene analysis, there are some differences in the degree of gene inhibition or induction under salt stress with different classifications. However, the expression of genes usually shows a significant upward trend, but the induced salt concentration is different, indicating that the molecular mechanism of *PtOSCAs* gene response to salt stress is slightly different.

A total of 16 *OSCA* gene sequences were identified in poplar genome. Based on the phylogenetic tree constructed with *Arabidopsis thaliana*, 16 PtOSCAs were divided into 4 groups, named I~IV group (Figure 1). Phylogenetic tree analysis showed that each group contained OSCA family genes from poplar and Arabidopsis. The results of gene structure analysis showed that all of them were located on chromosomes and contained conserved DUF221 domain. The analysis of doubling events and evolutionary selection pressure showed the formation and evolution of OSCA gene family in poplar. By analyzing the expression of *OSCA* gene under low and high concentration salt stress, it is found that there are the following three types: Type I, where the expression level increases under both low and high concentration salt stress; Type II, at low and high concentration salt stress, the expression level decreased under high concentration salt stress; Type III, the expression level showed an upward trend under low concentration salt stress, but the expression level showed a downward trend under high concentration salt stress. This study provides a reference for the functional analysis of *OSCA* gene and the genetic improvement of poplar.

3 Materials and Methods

3.1 Identification of OSCA gene family in poplar

In this study, the whole genome of *Populus tomentosa* was used as the analysis object to complete the identification and analysis of its OSCA gene family, and the related poplar genome and proteome data were downloaded based on the EnsemblPlants database. In the study, the Arabidopsis OSCA protein sequence has been identified as the seed sequence, and the poplar proteome data (e-value<1.0) has been searched and analyzed under the application of BLASTP (Imran et al., 2016). The redundant sequences in the gene sequences were removed manually, and the gene sequences were verified by InterProScan and SMART databases. The sequence of OSCA conserved domains (PF00056, PF02866 and PS00068) was identified as poplar OSCA gene sequence (Quevillon et al., 2005; Letunic and Peer, 2018). With the application of ExPASy software, the analysis of poplar OSCA gene was carried out, and the calculation mainly included protein sequence length, molecular weight and isoelectric point data. Based on WoLF PSOR and EuLoc online software, the prediction of subcellular localization of poplar OSCA protein was realized.

3.2 Phylogenetic analysis and classification of OSCA gene family in poplar

In this study, to achieve a detailed analysis of poplar genome, it is necessary to study 16 poplar OSCA protein sequences and combine them with the downloaded 15 Arabidopsis OSCA protein sequences to complete the construction of phylogenetic tree. In the actual research process, the Clustal W comparative analysis was carried out for the different protein sequences obtained above, and the phylogenetic tree was constructed based on the K-Nearest Neighbor under the default parameters in MEGA 7 software. The parameter settings in this process are

as follows: Possion model, pairwise deletion and 1000 bootstrap replications (Tao et al., 2018). Finally, according to the topological structure of the phylogenetic tree, 31 *OSCA* genes were divided into different groups.

3.3 Structure and conservative sequence analysis of OSCA gene family in poplar

In the process of poplar genome analysis, the corresponding structure information was extracted based on Perl program, and the corresponding *PtOSCAs* gene structure map was constructed based on GSDS online software (Guo et al., 2007). In the process of motif analysis of OSCA gene family protein sequence, the software MEME was used, and the corresponding parameters were as follows: amino acid length was 6~50; base order repeat number: arbitrary; the number threshold of motif discovery was 10 (Bailey et al., 2006).

3.4 Chromosome localization and doubling event analysis of poplar *OSCA* gene

The corresponding *PtOSCAs* gene location information can be obtained through the poplar genome annotation file, and the mapping of *OSCA* gene can also be completed under the application of MapChart software. Multiple Collinear Scan toolkit (MCScanX) software was used to realize the study of gene doubling events. In the process of fragment replication gene definition, the corresponding standard is as follows: the short sequence in the gene sequence is more than 70% of the long sequence; the similarity of different gene sequences was ensured to be more than 70% (Wang et al., 2012). If it is the number of intermediate isolation genes on the same chromosome, it must be ensured to be below one homologous gene, which is named tandem repeat gene. For the correlation analysis of *OSCA* gene doubling events, the image rendering was completed under the application of Circos software (Krzywinski et al., 2009). The Ks/Ka of the repeat gene pair were calculated by KaKs_Calculator 1.2.

3.5 Transcriptome analysis of poplar under salt stress

The expression levels of each member were extracted from transcriptome data of *Populus×euramericana cv.'74/76'* obtained by high-throughput sequencing in our previous research group after treatment with different salt concentrations (0, 3‰, 6‰), and $\log_2^{(1+RPKM)}$ treatment was performed for the obtained data. And the expression heatmap of each member under salt stress were plotted based on MEV 4.0 software (Saeed et al., 2003; Chen et al., 2018). Then, 16 *PtOSCAs* were classified, and their expression patterns were explored based on the difference of *OSCA* expression under different salt stress.

Authors' contributions

ZJ was the experimental designers and executor of this study. ZJ and CXH completed the data analysis and the writing of the first draft. ZJ, PDW, DAA, LXM participated in the experimental design, the analysis of the experimental results and the writing of the first draft. DAA and LXM participated in the experimental design, the analysis of the experimental results. SYH and YMS were the architects and responsible persons of this study, guiding experimental design, data analysis and paper writing and revision. All authors read and approved the final manuscript.

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