Evolutionary analysis of the OSCA gene family in sunflower (*Helianthus annuus L*) and expression analysis under NaCl stress

Feibiao Shan[1,2,3+], Yue Wu[3+], Ruixia Du[3], Qinfang Yang[3], Chunhui Liu[3], Yongxing Wang[3], Chun Zhang[1*], Yang Chen[1,3*]

1 Hetao College, Bayannur, China

2 Inner Mongolia Traditional Chinese & Mongolian Medical Research Institute, Hohhot, China

3 Bayannur Institute of Agricultural and Animal Sciences, Bayannur, China

Corresponding Author:

*Yang Chen/Assistant Professor/Research supervisor: chenyangrz@126.com

*Chun Zhang/Assistant Professor/Research supervisor: zhangchun6625@163.com

+ Feibiao Shan and Yue Wu contributed equally to this work.

Abstract: Hyperosmolality-gated calcium-permeable channels (OSCA) are Ca^{2+} nonselective cation channels that contain the calcium-dependent DUF221 domain, which plays an important role in plant response to stress and growth. However, the OSCA gene has not been fully identified and analyzed in sunflowers. In this study, we comprehensively analyzed the number, structure, collinearity, and phylogeny of the OSCA gene family in the sunflower, six Compositae species (*Arctium lappa, Chrysanthemum morifolium, Cichorium endivia, Cichorium intybus, Lactuca sativa var. Angustata,* and *Carthamus tinctorius*), and six other plants (soybean, *Arabidopsis thaliana*, rice, grape, and maize). The expression of the sunflower OSCA gene in nine different tissues, six different hormones, and NaCl stress conditions were analyzed based on transcriptome data and qRT–PCR. A total of 15 OSCA proteins, distributed on 10 chromosomes, were identified in the sunflower, and all of them were located in the endoplasmic reticulum. Using the phylogenetic tree, collinearity, gene structure, and motif analysis of the six Compositae species and six other plants, we found that the sunflower OSCA protein had only three subfamilies and lacked the Group 4 subfamily, which is conserved in the evolution of Compositae and subject to purification selection. The OSCA gene structure and motif analysis of the sunflower and six Compositae showed that there was a positive correlation between the number of motifs of most genes and the length of the gene, different subfamilies had different motifs, and the Group 4 subfamily had the smallest number of genes and the simplest gene structure. RNA-seq and qRT–PCR analysis showed that the expression levels of most OSCA genes in the sunflower changed to varying degrees under salt stress, and *HaOSCA2.6* and *HaOSCA3.1* were the most important in the sunflower's response to salt stress. The coexpression network of the sunflower genes under salt stress was constructed based on weighted gene co-expression network analysis (WGCNA). In conclusion, our findings suggest that the OSCA gene family

35 is conserved during the sunflower's evolution and plays an important role in salt tolerance. These results
36 will deepen our understanding of the evolutionary relationship of the sunflower OSCA gene family and
37 provide a basis for their functional studies under salt stress.

Introduction

39 The sunflower (*Helianthus annuus L.*) belongs to the Compositae family of annual herbs, which are
40 divided into edible the sunflower and oil sunflower (*Keeley et al., 2021*). Sunflower oil is one of the world's
41 four major oil crops (*Rele et al., 2003*). The main uses of edible sunflower are ingestion and food addition
42 (*Cao et al., 2003*). Because they have the advantages of simple cultivation and salt and drought tolerance,
43 sunflowers are known as a pioneer crop in saline-alkali land (*Keeley et al., 2021; Rele et al., 2003*). Planting
44 sunflowers on saline-alkali land has economic benefits as well as ecological benefits, such as the
45 desalination of the soil (*Keeley et al., 2021; Rele et al., 2003*). Acting as a second messenger, calcium ions
46 play an important role in the growth and development of plant cells (*Klimecka et al., 2007*). When a plant is
47 stimulated by the external environment, the Ca^{2+} concentration changes in the cell are regulated through
48 transport systems such as Ca^{2+} channels. Corresponding signals are generated, including changes in ion
49 transport, hormones, and protective enzyme systems, which then regulate the pore size, root hair
50 development, and absorption of water and nutrients in plant guard cells (*Shukla et al., 2014*). Both abiotic
51 and biotic stresses can induce plant stress resistance by activating the plant calcium ion pathway (*Shukla et
52 al., 2014; Ju et al., 2021*). The calcium ion pathway is very important for the transduction of calcium signals,
53 so the study of the calcium ion pathway has far-reaching significance when exploring the mechanism of
54 plant resistance regulation (*Liu et al., 1998; Xu et al., 2006; Xue et al., 2019*).

55 Calcium ion channels are mainly divided into hypotonic and hypertonic calcium ion channels, OSCA
56 belonging to the latter (*Murthy et al., 2018*). OSCA is a kind of hyperosmotic stress sensor (also called a
57 sensor protein) in *Arabidopsis thaliana*, as well as a Ca^{2+}-selective cation channel (*Zhang et al., 2018*). Other
58 common calcium channel proteins (such as depolarization-activated Ca^{2+}-permeable channels (DACCs),
59 hyperpolarization-activated Ca^{2+}-permeable channels (HACCs), and inositol 1,4,5-trisphosphate receptor
60 (IP3R)) have been systematically studied in plants, but there have been few studies on OSCA (*Swarbreck
61 et al., 2013; Gao et al., 2021*). Using protein-conserved domain analysis, it was found that OSCA gene
62 family proteins contained three conserved functional domains: late exocytosis (pfam13967), cytosolic
63 domain of 10TM putative phosphate transporter (pfam14703), and calcium-dependent channel
64 (pfam02714) (*Swarbreck et al., 2013; Gao et al., 2021*). In crops such as *Arabidopsis thaliana* and rice, the
65 OSCA gene family was divided into four subfamilies, all of which contained the DUF221 domain (*Yuan et
66 al., 2014; Li et al., 2015*). The DUF221 domain is essential, representative, and decisive for both the OSCA
67 gene family and represents the seven transmembrane domain regions of calcium-dependent channels that
68 function as calcium channels in osmotic sensing (*Kiyosue et al., 1994*). Previous studies used forward
69 genetics to screen *AtOSCA1* as an unknown hyperosmotic calcium ion permeability channel (*Yuan et al.,

70 *2014*). The results of this study indicated that *OSCA1* may be an osmotic sensor in *Arabidopsis thaliana*

71 (*Yuan et al., 2014; Zhang et al., 2020*). In rice, osmotic-related abiotic stresses (ABA, NaCl, and PEG)

72 differentially induced the expression of 10 OsOSCA genes, of which *OsOSCA-3.1* was the identified

73 *OsERD4* (early drought response) gene (*Li et al., 2015*). A drought-tolerant gene (*TaOSCA1.4*) was cloned

74 in wheat that belongs to the same gene family as *AtOSCA1.8* and *OsOSCA1.4* and is related to grain number

75 per ear and yield (*Zhai et al., 2020*). Thirteen GmOSCA genes in soybean (*Glycine max (Linn.) Merr*) were

76 associated with drought and alkali stress responses (*Yin et al., 2021*). A membrane-integral protein

77 (AtOSCA1.2) was obtained in *Arabidopsis thaliana* (*Liu et al., 2018*). This gene can increase the

78 concentration of intracellular calcium ions under hyperosmotic stress and increase the permeability of the

79 cell membrane to K^+ and Na^+. It has also been shown that *AtOSCA1.2* is an inherently mechanosensitive

80 pore-forming ion channel that may be necessary for osmotic sensing, and *AtOSCA1.1* and *AtOSCA3.1* (also

81 known as the *ERD4* gene) are mechanosensitive channels (*Liu et al., 2018; Zhang et al., 2018*).

82   OSCA family genes have been studied in a variety of plants and found to play potential roles in plant

83 stress resistance (*Li et al., 2015; Yin et al., 2021; Tong et al., 2021; Miao et al., 2022*). Following the

84 publication of the genomes of the sunflower and other Compositae plants, the evolution of this gene family

85 in Compositae has not been systematically studied. In this study, the number, structure, collinearity, and

86 phylogeny of the OSCA gene family were comprehensively analyzed using genomic data from the

87 sunflower and six Compositae species. RNA-sequencing (RNA-seq) and qRT–PCR were used to analyze the

88 expression of the sunflower OSCA gene in nine different tissues, six different hormones, and under NaCl

89 stress conditions. The coexpression network of OSCA under salt stress was constructed using WGCNA.

90 These results will further broaden our understanding of the evolutionary relationship of the sunflower OSCA

91 gene family and provide a basis for its functional study under salt stress.

Materials and Methods

93 **Plant material**

94   The sunflower salt-tolerant inbred line 19S05 (bred by Bayannur Institute of Agricultural and Animal

95 Sciences) was selected for this study. This material had a plant height of 200 cm, number of leaves was

96 approximately 30, leaves were green, without branches, and the inclination of the disc was 4. The diameter

97 of the disc was 20 cm, yellow flowers were ligulated, and the weight of 100 seeds was 16 g. The grains were

98 narrow oval, 2.00 cm long and 0.95 cm wide, with brown white edges, no streaks, and strong salt tolerance.

99 Selected and full 19S05 seeds were washed with water, surface sterilized with 3% H_2O_2 for 10 min, and

100 then placed in plastic pots filled with sterilized soil. After culturing four true leaves, a 200 mM NaCl stress

101 treatment was performed. Root samples were taken at 0 h of treatment and then at 1 h, 3 h, 6 h, 12 h, and 24 h

102 after treatment. Samples were then snap-frozen in liquid nitrogen (three biological replicates for each treatment)

103 and stored in a −80 °C refrigerator.

104 **Identification and cioinformatics analysis of OSCA genes**

105   *Helianthus annuus*, *Lactuca sativa var. Angustata, Gossypium hirsutum*, *Glycine max (Linn.) Merr*,

106 *Arabidopsis thaliana*, *Oryza sativa*, *Vitis vinifera,* and *Zea mays* genomic and proteomic data were downloaded

107 from the Ensembl Plants database (http://plants.ensembl.org/index.html) (*Bolser et al., 2016*). *Arctium lappa,*

108 *Cichorium endivia, and Cichorium intybus* genomic and proteomic data were downloaded from the NCBI

109 database (https://www.ncbi.nlm.nih.gov/). *Chrysanthemum morifolium* genomic and proteomic data were

110 downloaded from the *Chrysanthemum* genome database (http://www.amwayabrc.com/zh-cn/). *Carthamus*

111 *tinctorius L.* genomic and proteomic data were downloaded from the NGINX database

112 (http://118.24.202.236:11010/filedown/). Additionally, we used the OSCA gene domain Hidden Markov model

113 (PF02714) and HMMER software (http://hmmer.org/) to identify protein sequences validated in the NCBI-CDD

114 (https://www.ncbi.nlm.nih.gov/cdd/) database (*Mistry et al., 2021*). ExPASy software

115 (http://cn.expasy.org/tools) was used on the physicochemical data to calculate the protein, and EuLoc software

116 (http://euloc.mbc.nctu.edu.tw/) performed subcellular localization prediction (*Wilkins et al., 1999; Chang et al.,*

117 *2013*).

118 **Phylogenetic and collinear analysis of the OSCA gene family**

119   The OSCA protein sequences from *Helianthus annuus*, *Arctium lappa , Chrysanthemum morifolium,*

120 *Cichorium endivia, Cichorium intybus, Lactuca sativa var. Angustata, Carthamus tinctorius , Gossypium*

121 *hirsutum*, *Glycine max (Linn.) Merr*, *Arabidopsis thaliana*, *Oryza sativa*, *Vitis vinifera,* and *Zea mays* were used

122 for multiple sequence alignment. A phylogenetic tree was then constructed using the neighbor-joining method

123 (the bootstrap method value was set to 1,000 and the remaining parameters were set to default values) (*Hall,*

124 *2013*). The nwk file was obtained using MEGA11 software, and a phylogenetic tree of the OSCA gene was

125 made. The related collinearity map was constructed using the circle gene view function in TBtools (*Chen et al.,*

126 *2020; Wang et al., 2012*).

127 **Chromosomal location, gene structure, and motif analysis**

128   The chromosomal location information for the members of the sunflower OSCA gene family was extracted

129 using TBtools software, and the chromosomal location map was drawn using Mapchart software (*Chen et al.,*

130 *2020; Voorrips, 2002*). A phylogenetic tree of *Helianthus annuus*, *Arctium lappa L., Chrysanthemum*

131 *morifolium, Cichorium endivia, Cichorium intybus, Lactuca sativa var. Angustata,* and *Carthamus tinctorius L.*

132 OSCA sequences was constructed using MEGA11 software (*Wang et al., 2012*). OSCA motif types were

133 analyzed using Multiple Expectation maximizations for Motif Elicitation (MEME: http://meme-suite.org/)

134 (*Bailey et al., 2009*). Based on the mRNA sequence of the OSCA gene, the intron-exon structure was analyzed.

135 Graphs were made using TBtools software (*Chen et al., 2020*).

136 **Analysis of cis-acting elements of the sunflower OSCA gene**

137   The upstream 2,000 bp sequence of the sunflower OSCA gene was extracted, and the possible cis-acting

138 elements were predicted using the PlantCARE database

139 (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). After screening, the TBtools software was used

140 to map (*Chen et al., 2020*).

141 **RNA-seq analysis**

142   Transcriptome sequencing of different tissues of sunflower under different hormone and salt stress

143 conditions was downloaded from NCBI (SRP092742 and PRJNA866668). Fastp software was used for filtering,

144 removing adapters, and quality control of the raw data. Hisat2 software aligned the filtered data to the sunflower

145 reference gene, and StringTie software was used for expression quantification (*Kim et al., 2019; Pertea et al.,*

146 *2015*). Gene expression was assessed using the FPKM method, and the expression data were normalized to log10

147 (FPKM+1). Then, the expression heatmap was drawn with TBtools software (*Chen et al., 2018*).

148 **qRT–PCR**

149   Primers were designed in specific regions of the sunflower OSCA gene sequence using Primer 6.0 software.

150 The *HaActin* gene was chosen as the reference gene for qRT-PCR analysis (Table S1; *Li, 2010*). Root tissue

151 cDNA was used as the template to detect the expression of candidate genes by qRT–PCR, and each sample was

152 repeated three times. The total reaction system was 20 µl, qRT–PCR program: 94 °C for 30 s; 94 °C for 5 s, 60

153 °C for 15 s, 72 °C for 10 s for 38 cycles; and 4 °C for the end. Each group of data was replicated three times,

154 and the $2^{-\Delta\Delta Ct}$ method was used for relative quantitative analysis (*Tanino et al., 2017*).

155 **WGCNA**

156   The DEseq2 software package was used to identify differential genes under salt stress for WGCNA analysis.

157 We selected β=6 to process the original relation matrix in order to get a non-proportional adjacency matrix. The

158 adjacency matrix was further transformed into a topological overlap matrix (TOM). The minimum number of

159 genes in a module was 30, the threshold for merging similar modules was 0.25 (cutHeight=0.25), and the network

160 type was "signed" (type="signed" or networkType="signed"). Cytoscape-3.8.2 software was used for

161 visualization (*Shannon et al., 2003*). We performed Gene Ontology (GO) and Kyoto Encyclopedia of Genes

162 and Genomes (KEGG) analyses using the cluster Profiler software package in R language.

163 ## Result

164 **Identification of the OSCA gene family**

165   We systematically studied the changes in the copy number of the OSCA gene family during the sunflower's

166 evolution. First, using HMMsearch the OSCA gene was comprehensively searched from seven Compositae

167 species (*Helianthus annuus, Arctium lappa, Chrysanthemum morifolium, Cichorium endivia, Cichorium intybus,*

168 *Lactuca sativa,* and *Carthamus tinctorius)*. The search results were validated against the NCBI-CDD database.

169 *Helianthus annuus*, *Arctium lappa, Chrysanthemum morifolium, Cichorium endivia, Cichorium intybus, Lactuca*

170 *sativa,* and *Carthamus tinctorius* contained 15, 17, 20, 19, 19, 21, and 14 OSCA sequences, respectively. We

171 named them *HaOSCA1.1~HaOSCA3.1* according to the 15 sunflower sequences and the phylogenetic tree of 12

172 other plants. The protein encoded by sunflower OSCA family genes contained 648-838 amino acid residues, the

173 length of the open reading frame (ORF) was 1,947-2,517 bp, the theoretical isoelectric point was 7.61-9.94, and

174 the relative molecular weight was 73.42-96.62 kDa. The protein subcellular localization showed that HaOSCA

175 was localized to the endoplasmic reticulum, vacuole, chloroplast, golgi apparatus. and mitochondrion (Table 1).

176   Fifteen HaOSCA genes were distributed on 10 sunflower chromosomes (Chr03, Chr04, Chr05, Chr06,

177 Chr07, Chr09, Chr12, Chr15, Chr16, and Chr17) (Fig. 1). Among these, Chr07 and Chr16 staining

178 contained the most (three) OSCA genes. Except for Chr10, which contained two OSCA genes, the other

179 chromosomes contained only one OSCA gene.

180

181 **Evolutionary analysis of the OSCA gene family**

182   To better understand the evolutionary relationship of the sunflower OSCA family genes, we constructed a

183 phylogenetic tree of the full-length OSCA protein sequences of *Helianthus annuus, Gossypium hirsutum,*

184 *Glycine max (Linn.) Merr, Arabidopsis thaliana, Oryza sativa, Vitis vinifera,* and *Zea mays* (Fig. 2A). According

185 to the results, the evolutionary tree was divided into four groups. Compared with *Gossypium hirsutum, Glycine*

186 *max (Linn.) Merr, Arabidopsis thaliana, Oryza sativa, Vitis vinifera,* and *Zea mays*, there was no sunflower

187 OSCA protein in Group 4 (Fig, 2), which may have been caused by the loss of genes in the sunflower during the

188 evolution process. The number of OSCA proteins in Group 1 and Group 2 subgroups was consistent both in the

189 sunflower and in *Gossypium hirsutum, Glycine max (Linn.) Merr, Arabidopsis thaliana, Oryza sativa, Vitis*

190 *vinifera,* and *Zea mays* (Fig. 2B). The number of OSCA genes in sunflower, *Arabidopsis thaliana,* and *Glycine*

191 *max (Linn.) Merr* was basically the same, and the sunflower genes of the same branch were more closely related

192 to those in *Arabidopsis thaliana* and *Glycine max (Linn.) Merr*. Except for soybean and cotton, the other crops

193 in Group 3 all contained an OSCA sequence, which also indicated that the Group 3 subgroup of the OSCA gene

194 family was more conserved during the plant's evolution. Although Group 3 and Group 4 had relatively few

195 members, they were retained in the evolution of plants, suggesting that they may play important roles in

196 biological processes. However, the loss of members of sunflower Group 4 may have also been caused by

197 functional redundancy of genes, which requires further study.

198   To further explore the evolutionary relationship of the sunflower OSCA gene family, we selected the

199 sunflower OSCA gene as the core and constructed a colinear relationship between sunflower and *Gossypium*

200 *hirsutum, Glycine max (Linn.) Merr, Arabidopsis thaliana, Oryza sativa, Vitis vinifera,* and *Zea mays* OSCA

201 genes (Fig. 3). We found that the sunflower OSCA had 18 collinear gene pairs with *Arabidopsis thaliana*, 36

202 with *Gossypium hirsutum*, 12 with *Glycine max (Linn.) Merr*, eight with *Oryza sativa*, and 10 with *Vitis vinifera*.

203 There were 11 collinear gene pairs with *Zea mays* (Fig 3). The greatest collinearity between sunflower OSCA

204 and cotton may have been related to cotton having a larger genome and the largest number of OSCA genes. In

205 addition to cotton, the sunflower OSCA had greater collinearity with *Arabidopsis thaliana* and *Glycine max*

206 *(Linn.) Merr*, which was consistent with the results of the phylogenetic tree analysis.

207   To further study the evolutionary relationship of the sunflower OSCA gene family, we constructed a

208 phylogenetic tree based on the OSCA protein sequences of six Compositae species (*Arctium lappa L.,*

209 *Chrysanthemum morifolium, Cichorium endivia, Cichorium intybus, Lactuca sativa var. Angustata,* and

210 *Carthamus tinctorius L.*) and *Helianthus annuus* (Fig. 4A). Except for *Helianthus annuus* and *Carthamus*

211 *tinctorius L.*, which lacked the Group 4 subgroup, all Asteraceae contained four subgroups (Fig. 4A). The

212 *Cichorium endivia* and *Lactuca sativa var. Angustata* Group 4 subgroups contained three and four OSCA genes,
213 respectively, while *Arctium lappa L.*, *Chrysanthemum morifolium*, *Cichorium endivia*, and *Carthamus tinctorius*
214 *L* all contained one, which was consistent with the results from *Arabidopsis thaliana*, *Glycine max (Linn.) Merr*,
215 *Oryza sativa,* and *Zea mays* (Fig. 2B and Fig. 4B). Except for *Chrysanthemum morifolium* Group 3, which
216 contained four sequences, there was no difference in the subgroups of Group 1, Group 2, and Group 3 based on
217 Compositae.

218   We also constructed the collinear relationships for the OSCA of seven species of Compositae and
219 found that there were three pairs of HaOSCA (*HaOSCA1.1* and *HaOSCA2.1*, *HaOSCA1.2* and *HaOSCA2.2*,
220 *HaOSCA1.3* and *HaOSCA1.5*) with tandem repeats in sunflower genes (Fig 5A), indicating that these three
221 gene pairs may have been derived from tandem repeats. At the same time, five pairs of HaOSCA
222 (*HaOSCA1.5* and *HaOSCA1.6*, *HaOSCA2.2* and *HaOSCA2.1*, *HaOSCA1.2* and *HaOSCA1.1*, *HaOSCA1.8*
223 and *HaOSCA1.7*, *HaOSCA1.3* and *HaOSCA1.1*) showed very high identities (over 84.77%), implying that
224 they may have arisen from segments or whole genes (Fig. 5A). Additionally, the ratio of non-synonymous
225 to synonymous (Ka/Ks) between duplicate gene pairs was calculated, and the Ka/Ks values of all gene pairs
226 were less than 1, indicating that these gene pairs may have undergone purifying selection (Table S2).
227 *Helianthus annuus* had 32 collinear gene pairs with *Arctium lappa L.*, 32 with *Chrysanthemum morifolium*,
228 29 with *Cichorium endivia*, 31 with *Cichorium intybus*, and 39 with *Lactuca sativa var. Angustata,* and 18
229 collinear gene pairs with *Carthamus tinctorius L.*. In addition to having lower collinearity with *Carthamus*
230 *tinctorius*, *Helianthus annuus* OSCA had more collinearity with other Compositae species, which indicates
231 that the OSCA gene family was conserved in the evolution of Compositae. Moreover, *Helianthus annuus*
232 and *Lactuca sativa var. Angustata* had the most collinear gene pairs and were also close to *Lactuca sativa*
233 *var. Angustata* OSCA in the evolutionary tree, which indicates that the OSCA of *Helianthus annuus* and
234 *Lactuca sativa var. Angustata* may have the same biological function.

235 **Gene structure and motif analysis of the OSCA gene of Compositae**

236   To further understand the composition of the sunflower OSCA gene, we compared the OSCA gene structure
237 of seven Compositae species. All OSCA genes, except Group 4, contained introns (Fig. 6). Members of the same
238 subfamily had similar conserved motifs, and Group 4 had the simplest gene structure and least number of motifs
239 (Fig. 6). The Pfam website (http://pfam.xfam.org/family/pf02714#tabview=tab 0) showed that the RSN1_7TM
240 domain generally contained seven transmembrane domains. In this paper, motif 2, motif 3, motif 4, motif 6,
241 motif 7, motif 8, and motif 9 showed that they may contain multiples of the seven transmembrane domains. Only
242 a few OSCA genes (*CtOSCA1.5, CiOSCA2.2,* and *LsOSCA4.1*) contained a motif, although they were relatively
243 long. *CmOSCA2.2* was the longest and contained 10 exons, but it also contained eight motifs. The *LsOSCA4.4*
244 gene had the shortest length and contained one exon but only one motif (Motif 3). This indicates that the
245 distribution of OSCA gene motifs in Compositae was highly correlated with the length of the genes.

246 **Analysis of cis-acting elements in the promoter of the sunflower OSCA gene**

247   Transcription factors (TFs) are proteins that can bind to the promoter regions of genes in a sequence-

248 specific manner in order to regulate transcription, thereby regulating plant functions, including responses to

249 environmental factors, growth, and development (*Warren et al., 2002*). Analysis of the 2,000 bp upstream

250 sequence of the sunflower OSCA gene start codon showed that there were multiple stress and hormone

251 response elements in the upstream sequence of the HaOSCA gene, with different types and numbers (Fig 7).

252 Three main categories of cis elements were found in the promoter sequences of HaOSCA genes. The first

253 category was involved in phytohormones, such as abscisic acid (ABA), jasmonic acid (JA), auxin, gibberellins

254 (GA), and salicylic acid (SA). The second category was associated with stresses, such as low-temperature

255 responsiveness, defense, and stress responsiveness. The last category was mainly MYB binding sites.

256 Importantly, all 14 HaOSCA genes, except *HaOSCA1.8*, contained the JA-responsive element (TGACG-motif

257 and CGTCA-motif), and the ABA responsive element (ABRE) was found in almost all gene promoters (Table

258 S3). Interestingly, cis-acting elements were not found in the promoter region of *HaOSCA1.8*. These results

259 showed that HaOSCA may affect hormone signal responsiveness and stress adaptation. No cytokinin-

260 responsive elements were identified in the HaOSCA promoter regions.

261 **Expression analysis of the sunflower OSCA gene**

262   Gene expression patterns are usually closely related to gene functions. To gain insight into the expression

263 patterns of sunflower OSCA genes, we first analyzed the expression patterns of 15 sunflower OSCA genes in

264 nine tissues (Fig. 8A). The results showed that there were nine sunflower OSCAs (*HaOSCA1.1, HaOSCA1.2,*

265 *HaOSCA1.4, HaOSCA1.6, HaOSCA1.7, HaOSCA1.8, HaOSCA2.5, HaOSCA2.6,* and *HaOSCA3.1*) in pollen

266 and stamen. The expression was higher than in other tissues, and the remaining six had higher expression levels

267 in root, stem, leaves, bract, corolla, ovary, and ligament, which indicated that sunflower OSCA had a wide range

268 of tissue expression types.

269   Plant hormones play an important role in the processes of growth, development, and stress. We analyzed

270 the expression pattern of the sunflower OSCA gene induced by six hormones (ABA, BR, JA, GA, IAA, and SA)

271 (Fig. 8B). Six genes (*HaOSCA1.3, HaOSCA1.5, HaOSCA1.8, HaOSCA2.2, HaOSCA2.3,* and *HaOSCA2.4*) were

272 more strongly induced by ABA than other hormones, six genes (*HaOSCA1.2, HaOSCA1.4, HaOSCA1.6,*

273 *HaOSCA1.7, HaOSCA2.5,* and *HaOSCA2.6*) were more strongly affected by IAA than other hormones, and

274 HaOSCA1.1 was most strongly affected by JA.

275   Next, we analyzed the expression pattern of the sunflower OSCA gene under salt stress. All sunflower

276 OSCA gene expression levels changed after NaCl treatment (Fig. 8C). Seven OSCA genes (*HaOSCA1.1,*

277 *HaOSCA1.2, HaOSCA1.5, HaOSCA1.7, HaOSCA2.2, HaOSCA2.6,* and *HaOSCA3.1*) were upregulated, three

278 (*HaOSCA1.4, HaOSCA1.6,* and *HaOSCA2.5*) were significantly downregulated, and the expression levels of the

279 remaining five (*HaOSCA1.3, HaOSCA1.8, HaOSCA2.1, HaOSCA2.3,* and *HaOSCA2.4*) also changed, but not

280 significantly (Fig. 8C). These results indicated that the 10 OSCA genes (*HaOSCA1.1, HaOSCA1.2, HaOSCA1.4,*

281 *HaOSCA1.5, HaOSCA1.6, HaOSCA1.7, HaOSCA2.2, HaOSCA2.5, HaOSCA2.6,* and *HaOSCA3.1*) may play a

282 role in the sunflower's response to salt stress.

283 **qRT–PCR of the sunflower OSCA gene under salt stress**

284   With the increase of soil salinization, salt stress has become the most important abiotic stress faced by the

285 sunflowers. Based on the previous expression analysis, we found that ten genes (*HaOSCA1.1, HaOSCA1.2,*

286 *HaOSCA1.4, HaOSCA1.5, HaOSCA1.6, HaOSCA1.7, HaOSCA2.2, HaOSCA2.5, HaOSCA2.6,* and *HaOSCA3.*

287 *1*) may be involved in the response of sunflower to salt stress. qRT–PCR was used to study the expression

288 patterns of these 10 genes under NaCl stress. Compared with levels before stress (0 h), the expression of seven

289 genes (*HaOSCA1.1, HaOSCA1.2, HaOSCA1.5, HaOSCA1.6, HaOSCA2.2, HaOSCA2.6,* and *HaOSCA3.1*) was

290 significantly changed at different points in time during treatment (Fig 9). *HaOSCA1.6* was significantly

291 downregulated and the remaining six genes were significantly upregulated. The expression levels of *HaOSCA2.6*

292 and *HaOSCA3.1* reached more than a 10-fold change at 6 h of NaCl stress. These results suggest that *HaOSCA2.6*

293 and *HaOSCA3.1* may play an important role in the response of sunflower to salt stress.

294 **Protein interaction of sunflower OSCA gene**

295   Genes typically perform their biological functions and signal transduction pathways through interacting

296 networks, so studying the underlying interaction networks associated with gene families can provide a better

297 understanding of their functions. To elucidate the role of OSCA genes in salt stress in the sunflower, we used

298 WGCNA to analyze OSCA genes (*HaOSCA1.1, HaOSCA1.2, HaOSCA1.5, HaOSCA1.6, HaOSCA2.2,*

299 *HaOSCA2.6,* and *HaOSCA3.1*) and the salt coexpression network of all differentially expressed genes under

300 stress. Genes were clustered using dynamic shearing and divided into modules. By calculating the eigenvectors

301 of each module and merging similar modules, a total of seven gene coexpression modules were obtained (Fig.

302 10A). In this study, *OSCA* genes in each module were selected as core genes, and gene interaction network

303 diagrams were drawn using core genes and their interacting genes (Fig. 10B and Table S4). To further uncover

304 the function of the network, we performed an enrichment analysis of all interacting genes (Fig, 10C and D).

305 The GO enrichment results showed that the biological processes involved were mainly cellular component

306 organization or biogenesis, biological regulation, multicellular organismal process, response to stimulus and

307 cellular process, cellular components are extracellular regions, cells and extracellular regions, molecular

308 functions are transporter activities, antioxidant activities, transcription regulator activities, and molecular carrier

309 activity (Fig. 10C). The KEGG pathway was mainly alpha-linolenic acid metabolism, glycerolipid metabolism,

310 peroxisome, and tryptophan metabolism (Fig. 10D). This network further demonstrated the complex functions

311 and potential roles of the OSCA gene family in sunflower salt-stressed species. The results of this study lay a

312 foundation for further research on the functions and molecular mechanisms of these genes in the sunflower under

313 salt stress and provide a reference for further study of the sunflower OSCA gene family.

314 ## Discussion

315   Over their long evolutionary history, all living plants are thought to have undergone at least one or more

316 whole genome duplication (WGD) events, which provide opportunities for repeated genes to acquire functional

317 diversification, leading to more complex organisms. In this process of differentiation, the gene family produces

318 more members (*Zhang et al., 2018; Swarbreck et al., 2013*). Plant biologists have always been fascinated by

319 the structure, function, and evolution of genes, and the interactions and adaptations between the environment

320 and plants have been well studied *(Gao et al., 2021; Yuan et al., 2014)*. With the continuous publication of

321 plant genomes, the structure, function, and evolution of various gene families have been extensively studied

322 in plants, and these results can provide more insight into the origin, diversity, and biological function of

323 these gene families (*Kiyosue et al., 1994; Glasauer et al., 2014; Li et al., 2015; Tong et al., 2021; Miao et*

324 *al., 2022*). Asteraceae is made up of many different species, each with a rich morphological type that exhibits

325 extreme characteristics (*Badouin et al., 2017*). The evolution of the cultivated sunflower progressed in two

326 phases (domestication by native North Americans, followed by breeding according to traits related to modern

327 agricultural production) and have two complex WGD histories that provide an excellent opportunity to study the

328 relationship between gene family isolation and plant morphological changes (*Badouin et al., 2017*). In a

329 breakthrough study in 2014, the first hypertonic-induced Ca^{2+} conductive cation channel obtained from plants

330 was discovered, which was designated to reduce hypertonic-induced $[Ca^{2+}]$ increase1 (*OSCA1*) (*Yuan et al.,*

331 *2014*). *OSCA1* mutants exhibited impaired osmotic Ca^{2+} signaling in guard cells and root cells, as well as

332 weakened water transport regulation and root growth response under osmotic stress conditions (*Yuan et al.,*

333 *2014*). This work advanced the study of OSCA genes, leading to the identification of more OSCA gene families

334 (*Yuan et al., 2014*). The 15 sunflower OSCA genes obtained in this study were close to the number of OSCA

335 gene families in other crops, but much fewer than in soybeans and cotton, which may be related to the size of

336 the soybean and cotton genomes and the large number of replications within their OSCA family members. The

337 cell membrane (the dividing line between the internal and external environment of the cell) is the first to feel the

338 stress stimulus, and OSCA is a calcium channel with multiple transmembrane domains, which is consistent with

339 the subcellular structure predictions in this study (Table 1). This may be because when plants encounter

340 adversity, genes in cells will regulate multiple organs to, through the synthesis of organic matter, generate energy

341 and jointly resist adversity and stress, which needs to be further explored (*Yin et al., 2021; Miao et al., 2022*).

342 In *Arabidopsis thaliana*, two tandem repeat events occurred in the OSCA family (*Kiyosue et al., 1994*).

343 Moreover, tandem duplications of the OSCA gene family have been detected in rice, litchi, wheat, and soybean

344 species (*Li et al., 2015; Tong et al., 2021; Yin et al., 2021; Miao et al., 2022*). In this study, an in-depth

345 evolutionary analysis of the sunflower OSCA gene family was performed using the OSCA genes of 13 species

346 (Figs. 2 - 6). The HaOSCA evolutionary tree was subdivided into three subfamilies, which was inconsistent

347 with the OSCA family member evolutionary tree subfamily division of rice, soybean, corn, and other plants (*Li*

348 *et al., 2015; Tong et al., 2021; Ding et al., 2019; Wang et al., 2019*). HaOSCA has the closest evolutionary

349 tree clades and the largest number of collinear gene pairs to *Arabidopsis thaliana* and soybean, suggesting that

350 the research basis of *Arabidopsis thaliana* and soybean OSCA genes can be used to expand our understanding

351 of sunflower OSCA genes (*Li et al., 2015; Tong et al., 2021; Ding et al., 2019; Wang et al., 2019*). Three

352 pairs of tandem duplication genes were found in the sunflower OSCA gene family, which indicated that the

353 expansion and evolution of the sunflower OSCA gene family might be caused by tandem duplication. According

354   to the Ka/Ks analysis, the strong purifying selection signal also indicated the functional importance of the

355   sunflower OSCA gene. We also hypothesized that this may be because nonsynonymous substitutions generally

356   bring harmful traits and rarely result in an evolutionary advantage (*Swarbreck et al., 2013; Miao et al., 2022*).

357   The six Asteraceae species contained four subgroups, and only three of these subgroups (1, 2, and 3) were

358   included in the sunflower. Previous studies in tomatoes found that tomato OSCA family members were divided

359   into five subfamilies, which indicates that the number of genetic family evolutionary subfamilies may be greatly

360   related to crop growth habits (Wang et al., 2019). We found that sunflower OSCA had more collinearity with

361   other Asteraceae plants and less collinearity with Carthamus tinctorius, indicating that the OSCA gene family

362   was conserved during the evolution of Asteraceae. Sunflower and *Lactuca sativa var. Angustata* had the most

363   colinear gene pairs, and the evolutionary tree was also closer to *Lactuca sativa var. Angustata* OSCA, suggesting

364   that sunflower may have the same biological function as *Lactuca sativa var. Angustata* OSCA. Combined with

365   the evolutionary tree, the number of CDS coding regions of family members from the same subfamily was

366   similar, but the number of different subfamilies varied greatly, and it is speculated that the functions performed

367   by subfamilies may be different.

368   The functions of multiple OSCA genes involved in regulating plant growth, development, and various stress

369   responses have been verified, and the roles played by different tissues and growth stages are different (*Zhang et*

370   *al., 2018; Swarbreck et al., 2013; Gao et al., 2021; Kiyosue et al., 1994; Yin et al., 2021; Miao et al., 2022*).

371   In this study, cis-acting elements in the promoter region of the sunflower OSCA gene were analyzed, and we

372   found that cis-acting elements in the binding sites of folic ABA and JA-bound cis-acting elements were the most

373   abundant. Through expression analysis, we also found that more HaOSCA genes were induced by ABA and JA

374   (Fig. 8B). A growing number of studies have found that ABA is the most important hormone for plant abiotic

375   stress, and JA also plays an important role in this process (*Wang et al., 2020; Baldoni et al., 2015*). ABA has

376   been found to activate the phosphorylation membrane of SnRKs bound to NADPH oxidase RBOHF, increasing

377   the production of H_2O_2 in apoplasts (*Umezawa et al., 2009*). Salt-induced ABA and Ca^{2+} signaling activates

378   RBOHF activity through *SnRK2.6* and *CIPK11/26* signaling modules, while *ABI1* is inhibitory. ABA, ROS, and

379   Ca^{2+} exhibit complex signal crosstalk to control plant responses to salt stress (*Quan et al., 2007*). JA levels are

380   elevated and JA signaling is activated under salt stress (*Chen et al., 2016*). The F-box protein COI1, also known

381   as the JA receptor, forms complexes with SKP1 and CULLIN1 to mediate JAZ degradation (*Valenzuela et al.,*

382   *2016*). When JAZ is removed, inhibited transcription factors such as MYC activate the expression of JA response

383   genes. Activation of JA signaling under salt stress eventually leads to inhibition of primary root growth.

384   Exogenous JA can mitigate salt toxicity by maintaining ROS or ionic homeostasis (*Valenzuela et al., 2016*).

385   These combined results hint at the complexity and importance of the sunflower OSCA gene in the coordination

386   of growth and adversity.

387   The yield and quality of the sunflower are susceptible to various biotic and abiotic stresses (*Dar et al.,*

388   *2021; Gogna et al., 2019*). Abiotic stresses such as salinity and temperature often have a greater impact on

389   plants (*Gong et al., 2020; Verma et al., 2016*). Although sunflowers are not halophytes, they have a strong

390   ability to tolerate salt and alkali (*Keeley et al., 2021; Rele et al., 2003*). This candidate gene for mining salt
391   tolerance-related genes is a good research model. The soybean OSCA gene family generally responds to abiotic
392   stress (saline-alkali), and nine OSCA genes were also upregulated or downregulated in rice abiotic stress
393   treatments (*Li et al., 2017*). In the sunflower, we found that the expression levels of *HaOSCA1.1, HaOSCA1.2,*
394   *HaOSCA1.5, HaOSCA1.6, HaOSCA2.2, HaOSCA2.6,* and *HaOSCA3.1* all changed significantly at different
395   time points during NaCl treatment. Of these, only *HaOSCA1.6* was significantly downregulated (Fig. 9). The
396   expression levels of *HaOSCA2.6* and *HaOSCA3.1* reached more than a 10-fold change at 6 h of NaCl stress.
397   These results suggest that *HaOSCA2.6* and *HaOSCA3.1* may be the most important in the sunflower's response
398   to salt stress.

399   Studies on other plants have shown that most processes related to plant growth, development, and
400   physiology are regulated by networks of protein–protein interactions (*Yuan et al., 2014; Zhang et al., 2020;*
401   *Liu et al., 2018*). Building interaction networks between proteins is important for understanding how proteins
402   work in biological systems. It is of great significance to understand the growth and development of plants,
403   biological signals, and response mechanism of energy metabolism in some specific physiological states, as well
404   as to understand the functional connections between proteins. We used WGCNA to analyze the coexpression
405   network of sunflower OSCA genes and differentially expressed genes under salt stress (Fig. 10). Using the
406   KEGG pathway enrichment analysis of the interacting genes, we found that the main enrichments were in the
407   alpha-linolenic acid metabolism, glycerolipid metabolism, and peroxisome and tryptophan metabolism pathways
408   (Fig. 10D). In *Arabidopsis thaliana*, alpha-linolenic acid metabolism has been found to be important in
409   synthesizing JA (*Valenzuela et al., 2016*). *AtLOX3* is involved in the process of *Arabidopsis thaliana* salt stress
410   (*Kilaru et al., 2011*). Constitutive overexpression of AOC in wheat led to elevated JA levels and also confirmed
411   JA involvement in salt stress response (*Kilaru et al., 2011*). The function of JAZ congeners in salt stress
412   response has been demonstrated in many plant species (*Valenzuela et al., 2016*). In addition, the bHLH factor
413   RICE SALT SENSITIVE3 (*RSS3*)/*OsbHLH094* has been found to interact with JAZ to regulate salt tolerance
414   (*Toda et al., 2013*). In addition, overexpression of *OsbHLH148* in rice mutants induced high expression of
415   OsDREB and OsJAZ under drought stress (*Seo et al., 2011*). Similarly, overexpression of *OsJAZ9* significantly
416   improved the salinity and drought tolerance of rice (*Seo et al., 2011*). However, whether OSCA relies on the JA
417   pathway to improve the salinity tolerance of plants has not been reported, but provides a reference for our future
418   research. In conclusion, the expression analysis of sunflower OSCA genes under salt stress and the construction
419   of a coexpression network will provide clues to further study the salt tolerance of sunflower.

Conclusion

421   In this study, a systematic evolutionary analysis of sunflower OSCA genes was performed using 13 plant
422   OSCA genes, and the sunflower OSCA gene family was found to be conserved in the evolution of Compositae.
423   The analysis of the expression patterns of six hormones and salt stress in nine tissues showed that the OSCA
424   gene played an important role in regulating the response of sunflower to salt stress, and *HaOSCA2.6* and

425   *HaOSCA3.1* were the most important in the sunflower's response to salt stress. The coexpression network of the

426   sunflower OSCA gene under salt stress was also constructed based on WGCNA. In this study, the systematic

427   evolution and expression analysis of the sunflower OSCA gene family was carried out for the first time, and

428   provided clues for further research on the role of sunflower OSCA genes in salt-tolerant species.

429

430   Table S1. All primers used in this study.

431   Fig S1. Distribution of the OSCA domain in the OSCA proteins of sunflower.

432   Table S2. Ka/Ks ratios of repeated OSCA gene pairs in sunflower.

433   Table S3. Cis-acting element analysis of OSCA family members in sunflower.

434   Table S4. Co-expression network genes of sunflower under NaCl treatment.

435

References

437   **Badouin H, Gouzy J, Grassa CJ, Murat F, Staton SE, Cottret L, Lelandais-Brière C, Owens GL,**

438   **Carrère S, Mayjonade B, Legrand L, Gill N, Kane NC, Bowers JE, Hubner S, Bellec A, Bérard**

439   **A, Bergès H, Blanchet N, Boniface MC, Brunel D, Catrice O, Chaidir N, Claudel C, Donnadieu**

440   **C, Faraut T, Fievet G, Helmstetter N, King M, Knapp SJ, Lai Z, Le Paslier MC, Lippi Y,**

441   **Lorenzon L, Mandel JR, Marage G, Marchand G, Marquand E, Bret-Mestries E, Morien E,**

442   **Nambeesan S, Nguyen T, Pegot-Espagnet P, Pouilly N, Raftis F, Sallet E, Schiex T, Thomas J,**

443   **Vandecasteele C, Varès D, Vear F, Vautrin S, Crespi M, Mangin B, Burke JM, Salse J, Muños**

444   **S, Vincourt P, Rieseberg LH, Langlade NB. 2017.** The sunflower genome provides insights into

445   oil metabolism, flowering and Asterid evolution. *Nature* **546(7656)**:148-152.

446   **Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Ren J, Li WW, Noble WS. 2009.**

447   MEME SUITE: tools for motif discovery and searching. *Nucleic Acids Res* **37**:W202-8.

448   **Baldoni E, Genga A, Cominelli E. 2015.** Plant MYB Transcription Factors: Their Role in Drought

449   Response Mechanisms. *Int J Mol Sci* **13**;16(7):15811-51.

450   **Bolser D, Staines DM, Pritchard E, Kersey P. 2016.** Ensembl Plants: Integrating Tools for Visualizing,

451   Mining, and Analyzing Plant Genomics Data. *Methods Mol Biol* **1374**:115-40.

452   **Cao L, Yang JX, Guo JJ, Guo JM, Zheng GD, Lu YF. 2018.** Effect of Fertilizers on Cadmium Uptake

453   and Accumulation by Sunflowers. *Huan Jing Ke Xue* **8**;39(11):5189-5197.

454   **Chang TH, Wu LC, Lee TY, Chen SP, Huang HD, Horng JT.** 2013. EuLoc: a web-server for

455   accurately predict protein subcellular localization in eukaryotes by incorporating various features of

456   sequence segments into the general form of Chou's PseAAC. *J Comput Aided Mol Des* **27(1)**:91-103.

457   **Chen C, Chen H, Zhang Y, Thomas HR, Frank MH, He Y, Xia R. 2020.** TBtools: An Integrative

458   Toolkit Developed for Interactive Analyses of Big Biological Data. *Mol Plant* **3**;13(8):1194-1202.

459   **Chen X, Zhang X, Jia A, Xu G, Hu H, Hu X, Hu L. 2016.** Jasmonate mediates salt-induced nicotine

460   biosynthesis in tobacco (Nicotiana tabacum L.). *Plant Divers* **16**;38(2):118-123.

461 **Chen S, Zhou Y, Chen Y, Gu J. 2018.** fastp: an ultra-fast all-in-one FASTQ preprocessor.

462   *Bioinformatics* **1**;34(17):i884-i890.

463 **Dar JS, Cheema MA, Rehmani MIA, Khuhro S, Rajput S, Virk AL, Hussain S, Bashir MA,**

464   **Alghanem SM, Al-Zuaibr FM, Ansari MJ, Hessini K. 2021.** Potassium fertilization improves

465   growth, yield and seed quality of sunflower (Helianthus annuus L.) under drought stress at different

466   growth stages. *PLoS One* **20**;16(9):e0256075.

467 **Ding S, Feng X, Du H, Wang H. 2019.** Genome-wide analysis of maize OSCA family members and

468   their involvement in drought stress. *PeerJ* **11**;7:e6765.

469 **Gao T, Yang P, Fu D, Liu M, Deng X, Shao M, Liao J, Jiang H, Li X. 2021.** The protective effect of

470   allicin on myocardial ischemia-reperfusion by inhibition of Ca2+ overload-induced cardiomyocyte

471   apoptosis via the PI3K/GRK2/PLC-γ/IP3R signaling pathway. *Aging (Albany NY)* **3**;13(15):19643-

472   19656.

473 **Glasauer SM, Neuhauss SC. 2014.** Whole-genome duplication in teleost fishes and its evolutionary

474   consequences. *Mol Genet Genomics* **289(6)**:1045-60.

475 **Gogna M, Bhatla SC. 2019.** Biochemical mechanisms regulating salt tolerance in sunflower. *Plant*

476   *Signal Behav* **14(12)**:1670597.

477 **Gong Z, Xiong L, Shi H, Yang S, Herrera-Estrella LR, Xu G, Chao DY, Li J, Wang PY, Qin F, Li**

478   **J, Ding Y, Shi Y, Wang Y, Yang Y, Guo Y, Zhu JK. 2020.** Plant abiotic stress response and

479   nutrient use efficiency. *Sci China Life Sci* **63(5)**:635-674.

480 **Hall BG. 2013.** Building phylogenetic trees from molecular data with MEGA. Mol Biol Evol 30(5):1229-

481   35.

482 **Ju, C., Zhang, Z., Deng, J., Miao, C., Wang, Z., Wallrad, L., Javed, L., Fu, D., Zhang, T., Kudla, J.,**

483   **Gong, Z., and Wang, C. 2021.** Ca2+-dependent successive phosphorylation of vacuolar transporter

484   MTP8 by CBL2/3-CIPK3/9/26 and CPK5 contributes to manganese homeostasis inArabidopsis.

485   *Mol. Plant* **S1674-2052(21)**:00467-6.

486 **Keeley SC, Cantley JT, Gallaher TJ. 2021.** The "evil tribe" spreads across the land: A dated molecular

487   phylogeny provides insight into dispersal, expansion, and biogeographic relationships within one of

488   the largest tribes of the sunflower family (Vernonieae: Compositae). *Am J Bot* **108(3)**:505-519.

489 **Kim D, Paggi JM, Park C, Bennett C, Salzberg SL. 2019.** Graph-based genome alignment and

490   genotyping with HISAT2 and HISAT-genotype. *Nat Biotechnol* **37(8)**:907-915.

491 **Kiyosue T, Yamaguchi-Shinozaki K, Shinozaki K. 1994.** Cloning of cDNAs for genes that are early-

492   responsive to dehydration stress (ERDs) in Arabidopsis thaliana L.: identification of three ERDs as

493   HSP cognate genes. *Plant Mol Biol* **25(5)**:791-8.

494 **Kilaru A, Herrfurth C, Keereetaweep J, Hornung E, Venables BJ, Feussner I, Chapman KD. 2011.**

495   Lipoxygenase-mediated oxidation of polyunsaturated N-acylethanolamines in Arabidopsis. *J Biol*

496 *Chem* **29**;286(17):15205-14.

497 **Klimecka M, Muszyńska G. 2007.** Structure and functions of plant calcium-dependent protein kinases.
498   *Acta Biochim Pol* **54(2)**:219-33.

499 **Li Jianwei, Yang Junkai, Jia Bowei, Sun Mingzhe, Liu Yu, Yin Kuide, Sun Xiaoli. 2017.** Evolution
500   and expression analysis of OSCA gene family in soybean genome.*Chinese Journal of Oil Crops*
501   **39(5)**:589-599

502 **Li K, Brownley A. 2010.** Primer design for RT-PCR. Methods Mol Biol 630:271-99.

503 **Li Y, Yuan F, Wen Z, Li Y, Wang F, Zhu T, Zhuo W, Jin X, Wang Y, Zhao H, Pei ZM, Han S.**
504   **2015.** Genome-wide survey and expression analysis of the OSCA gene family in rice. *BMC Plant*
505   *Biol* **26**;15:261.

506 **Liu X, Wang J, Sun L. 2018.** Structure of the hyperosmolality-gated calcium-permeable channel
507   OSCA1.2. *Nat Commun* **29**;9(1):5060.

508 **Liu, J. and Zhu, J.K. 1998.** A calcium sensor homolog required for plant salt tolerance. Science
509   (80).280: 1943–1945.

510 **Miao S, Li F, Han Y, Yao Z, Xu Z, Chen X, Liu J, Zhang Y, Wang A. 2022.** Identification of OSCA
511   gene family in Solanum habrochaites and its function analysis under stress. *BMC Genomics*
512   **1**;23(1):547.

513 **Mistry J, Chuguransky S, Williams L, Qureshi M, Salazar GA, Sonnhammer ELL, Tosatto SCE,**
514   **Paladin L, Raj S, Richardson LJ, Finn RD, Bateman A. 2021.** Pfam: The protein families
515   database in 2021. *Nucleic Acids Res* **8**;49(D1):D412-D419.

516 **Murthy SE, Dubin AE, Whitwam T, Jojoa-Cruz S, Cahalan SM, Mousavi SAR, Ward AB,**
517   **Patapoutian A. 2018.** OSCA/TMEM63 are an Evolutionarily Conserved Family of Mechanically
518   Activated Ion Channels. *Elife* **1**;7:e41844.

519 **Pertea M, Pertea GM, Antonescu CM, Chang TC, Mendell JT, Salzberg SL. 2015.** StringTie enables
520   improved reconstruction of a transcriptome from RNA-seq reads. *Nat Biotechnol* **33(3)**:290-5.

521 **Quan R, Lin H, Mendoza I, Zhang Y, Cao W, Yang Y, Shang M, Chen S, Pardo JM, Guo Y. 2007.**
522   SCABP8/CBL10, a putative calcium sensor, interacts with the protein kinase SOS2 to protect
523   Arabidopsis shoots from salt stress. *Plant Cell* **19(4)**:1415-31.

524 **Rele AS, Mohile RB. 2003.** Effect of mineral oil, sunflower oil, and coconut oil on prevention of hair
525   damage. *J Cosmet Sci* **54(2)**:175-92.

526 **Seo JS, Joo J, Kim MJ, Kim YK, Nahm BH, Song SI, Cheong JJ, Lee JS, Kim JK, Choi YD. 2011.**
527   OsbHLH148, a basic helix-loop-helix protein, interacts with OsJAZ proteins in a jasmonate
528   signaling pathway leading to drought tolerance in rice.*Plant J* **65(6)**:907-21.

529 **Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker**
530   **T. 2003.** Cytoscape: a software environment for integrated models of biomolecular interaction
531   networks. *Genome Res* **13(11)**:2498-504.

532   **Shukla D, Huda KM, Banu MS, Gill SS, Tuteja R, Tuteja N. 2014.** OsACA6, a P-type 2B Ca(2+)
533   ATPase functions in cadmium stress tolerance in tobacco by reducing the oxidative stress load.
534   *Planta* **240(4)**:809-24.
535   **Swarbreck SM, Colaço R, Davies JM. 2013.** Plant calcium-permeable channels. *Plant Physiol*
536   **163(2)**:514-22.
537   **Tanino Y, Kodama M, Daicho H, Miyauchi Y, Yasumoto T, Yamad**a Y. 2017. Selection of laboratory
538   procedures to detect toxigenic by the 2-step method. *Rinsho Biseibutshu Jinsoku Shindan Kenkyukai*
539   *Shi* **27(1)**:9–14.
540   **Toda Y, Yoshida M, Hattori T, Takeda S. 2013.** RICE SALT SENSITIVE3 binding to bHLH and JAZ
541   factors mediates control of cell wall plasticity in the root apex. *Plant Signal Behav* **8(11)**:e26256.
542   **Tong K, Wu X, He L, Qiu S, Liu S, Cai L, Rao S, Chen J. 2021.** Genome-Wide Identification and
543   Expression Profile of OSCA Gene Family Members in Triticum aestivum L. *Int J Mol Sci*
544   **31**;23(1):469.
545   **Umezawa T, Sugiyama N, Mizoguchi M, Hayashi S, Myouga F, Yamaguchi-Shinozaki K, Ishihama**
546   **Y, Hirayama T, Shinozaki K. 2009.** Type 2C protein phosphatases directly regulate abscisic acid-
547   activated protein kinases in Arabidopsis. *Proc Natl Acad Sci U S A* **106(41)**:17588-93.
548   **Valenzuela CE, Acevedo-Acevedo O, Miranda GS, Vergara-Barros P, Holuigue L, Figueroa CR,**
549   **Figueroa PM. 2016.** Salt stress response triggers activation of the jasmonate signaling pathway
550   leading to inhibition of cell elongation in Arabidopsis primary root. *J Exp Bot* **67(14)**:4209-20.
551   **Verma V, Ravindran P, Kumar PP. 2016.** Plant hormone-mediated regulation of stress responses. *BMC*
552   *Plant Biol* **14**;16:86.
553   **Voorrips RE. 2002.** MapChart: software for the graphical presentation of linkage maps and QTLs. *J*
554   *Hered* **93(1)**:77-8.
555   **Wang Aoxue, Zhang Kewei, Zhang Yao, Chen Xiuling, Liu Jiayin. 2019.** Identification of tomato
556   OSCA gene family and expression analysis under different stress conditions. *Journal of Northeast*
557   *Agricultural University* **50(01)**:19-28.
558   **Wang J, Song L, Gong X, Xu J, Li M. 2020.** Functions of Jasmonic Acid in Plant Regulation and
559   Response to Abiotic Stress. *Int J Mol Sci* **20**;21(4):1446.
560   **Wang Y, Tang H, Debarry JD, Tan X, Li J, Wang X, Lee TH, Jin H, Marler B, Guo H, Kissinger**
561   **JC, Paterson AH. 2012.** MCScanX: a toolkit for detection and evolutionary analysis of gene
562   synteny and collinearity. *Nucleic Acids Res* **40(7)**:e49.
563   **Warren AJ**. 2002. Eukaryotic transcription factors. Curr Opin Struct Biol 12(1):107-14.
564   **Wilkins MR, Gasteiger E, Bairoch A, Sanchez JC, Williams KL, Appel RD, Hochstrasser DF. 1999.**
565   Protein identification and analysis tools in the ExPASy server. *Methods Mol Biol* **112**:531-52.
566   **Xu, J., Li, H.D., Chen, L.Q., Wang, Y., Liu, L.L., He, L., and Wu, W.H. 2006.** A Protein Kinase,
567   Interacting with Two Calcineurin B-like Proteins, Regulates K+Transporter AKT1 inArabidopsis.

568   *Cell* **125**: 1347–1360.

569   **Xue Y, Zhao H, Wang HN, Crawford BM, Johnson DM, Hu C, Pei C, Zhou W, Swift GB, Zhang H,**

570   **Vo-Dinh T, Hu Z, Siedow JN, Pei ZM. 2019.** Plant cell-surface GIPC sphingolipids sense salt to

571   trigger Ca2+influx. *Nature* **572**: 341–346

572   **Yin L, Zhang M, Wu R, Chen X, Liu F, Xing B. 2021.** Genome-wide analysis of OSCA gene family

573   members in Vigna radiata and their involvement in the osmotic response. *BMC Plant Biol*

574   **7**;21(1):408.

575   **Yuan F, Yang H, Xue Y, Kong D, Ye R, Li C, Zhang J, Theprungsirikul L, Shrift T, Krichilsky B,**

576   **Johnson DM, Swift GB, He Y, Siedow JN, Pei ZM. 2014.** OSCA1 mediates osmotic-stress-evoked

577   Ca2+ increases vital for osmosensing in Arabidopsis. *Nature* **16**;514(7522):367-71.

578   **Zhai Y, Wen Z, Han Y, Zhuo W, Wang F, Xi C, Liu J, Gao P, Zhao H, Wang Y, Wang Y, Han S.**

579   **2020.** Heterogeneous expression of plasma-membrane-localised OsOSCA1.4 complements osmotic

580   sensing based on hyperosmolality and salt stress in Arabidopsis osca1 mutant. *Cell Calcium*

581   **91**:102261.

582   **Zhang M, Wang D, Kang Y, Wu JX, Yao F, Pan C, Yan Z, Song C, Chen L. 2018.** Structure of the

583   mechanosensitive OSCA channels. *Nat Struct Mol Biol* **25(9)**:850-858.

584   **Zhang S, Wu QR, Liu LL, Zhang HM, Gao JW, Pei ZM. 2020.** Osmotic stress alters circadian

585   cytosolic Ca2+ oscillations and OSCA1 is required in circadian gated stress adaptation. *Plant Signal*

586   *Behav* **1**;15(12):1836883.

Figure 1

Fig. 1

Chromosomal location of the sunflower OSCA gene.
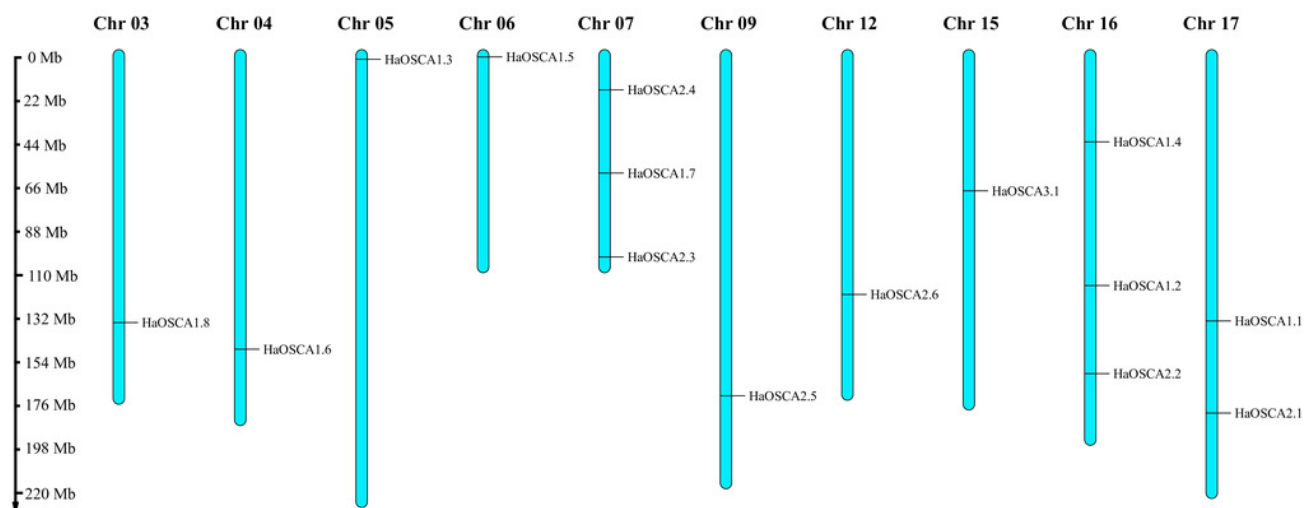
Figure 2

Fig. 2

A) OSCA gene tree of sunflower, cotton, soybean, *Arabidopsis thaliana*, rice, grape, and maize. The different colors indicate different groups (1, 2, 3 and 4) of OSCA genes, different traits represent OSCA genes of different plants. B) Sunflower, cotton, soybean, *Arabidopsis thaliana*, rice, grape, and maize OSCA gene evolutionary tree. The number of genes in each group.

Figure 3

Fig. 3

Collinear relationship of OSCA genes in sunflower, cotton, soybean, *Arabidopsis thaliana*, rice, grape, and maize. The different colors represent a collinear relationship between different plants.
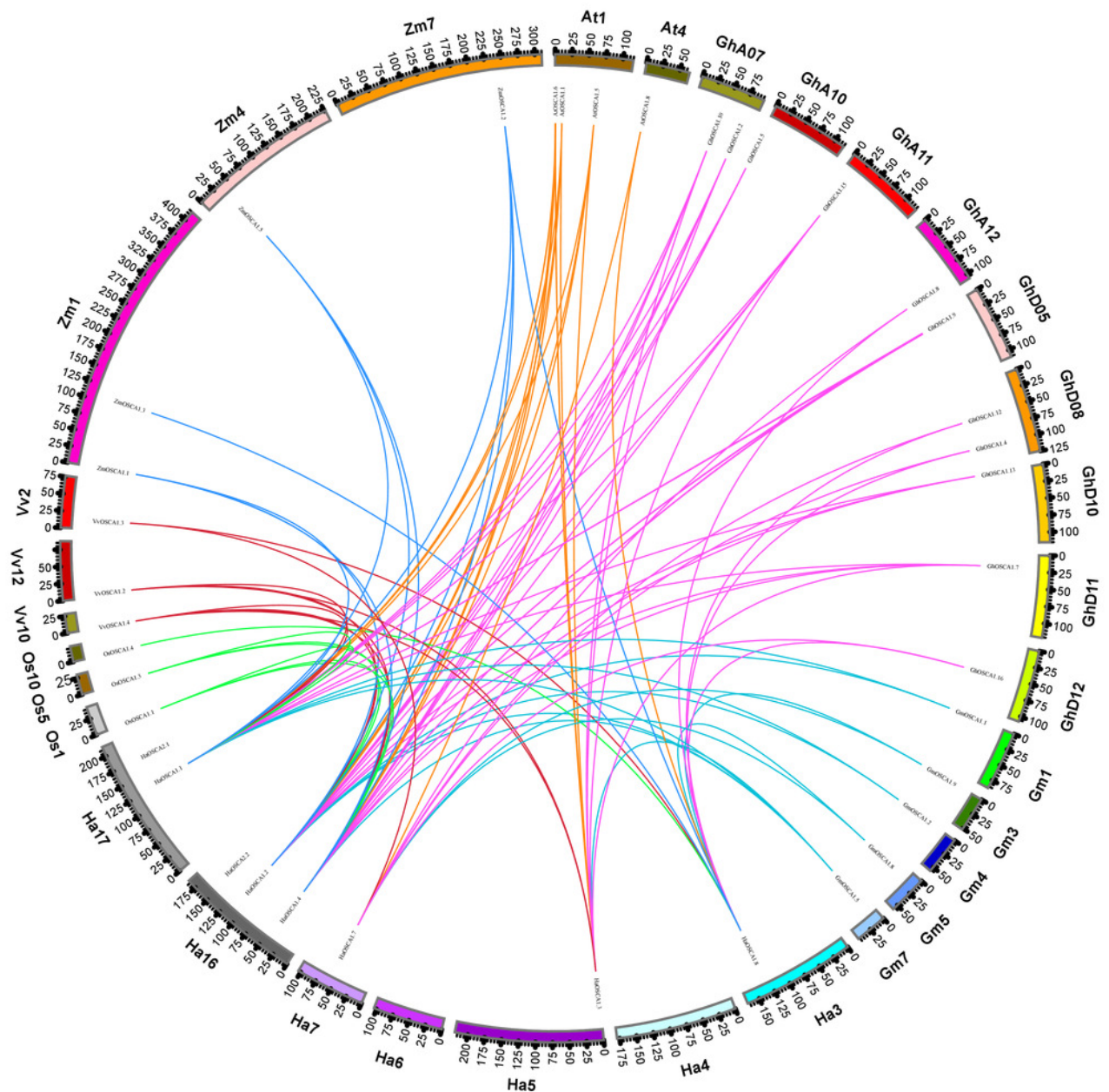
Figure 4

Fig. 4

A) OSCA gene tree of sunflower, *Arctium lappa, Chrysanthemum morifolium, Cichorium endivia, Cichorium intybus, Lactuca sativa,* and *Carthamus tinctorius*. The different colors indicate different groups (1, 2, 3 and 4) of OSCA genes, different traits represent OSCA genes of different plants. B) Sunflowers, *Arctium lappa, Chrysanthemum morifolium, Cichorium endivia,* and Cichori.

Figure 5

Fig. 5

Collinearity of sunflowers, *Arctium lappa, Chrysanthemum morifolium, Cichorium endivia, Cichorium intybus, Lactuca sativa,* and *Carthamus tinctorius* OSCA genes. The different colors represent a collinear relationship between different plants.

Figure 6

Fig. 6

Phylogenetic tree, gene structure, and conserved motif analysis of the OSCA gene family of Compositae. Different motifs are displayed in different colored boxes as indicated on the right side.
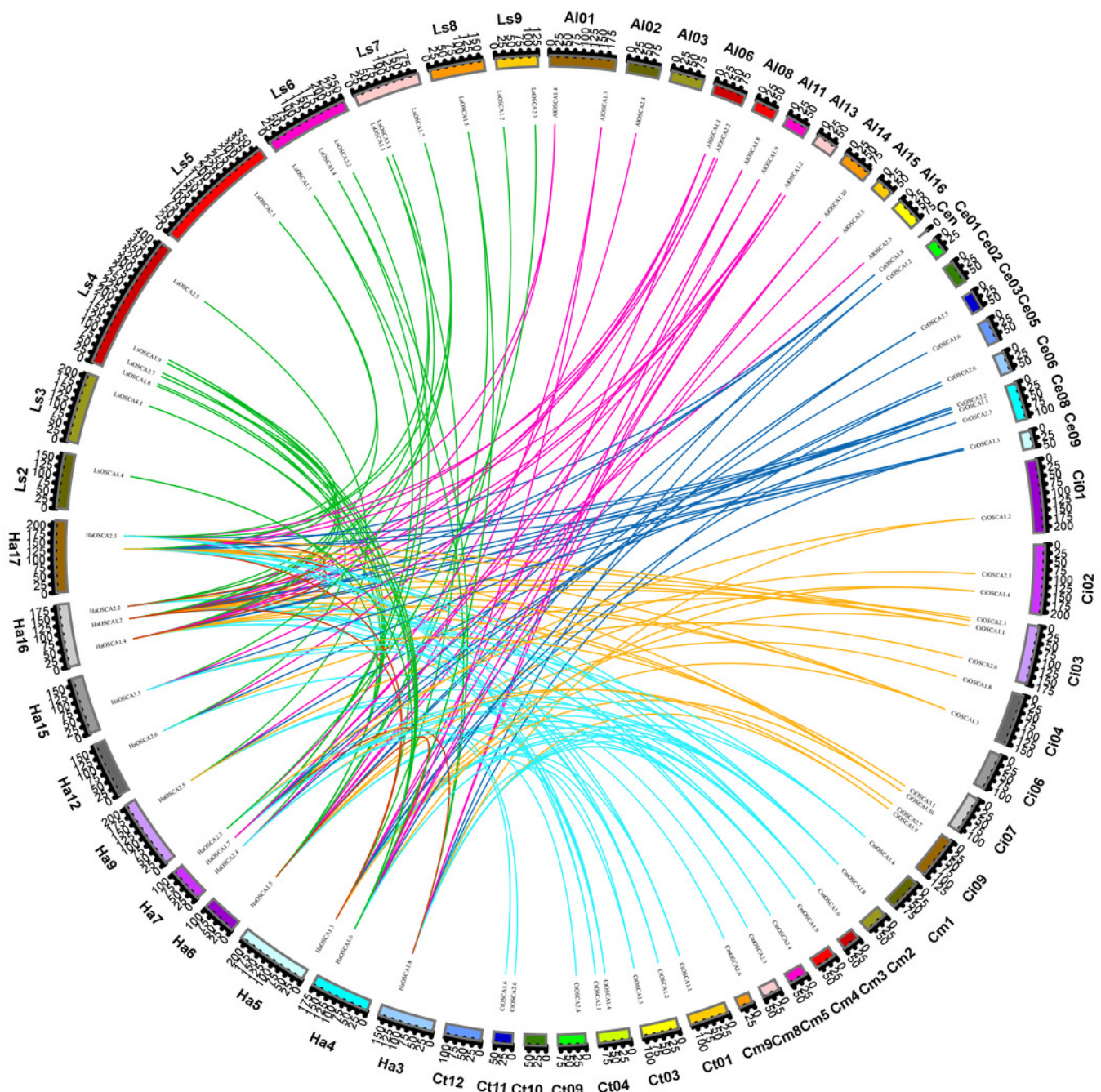
Figure 7

Fig. 7

Analysis of homeopathic elements in the promoters of the sunflower OSCA gene family.
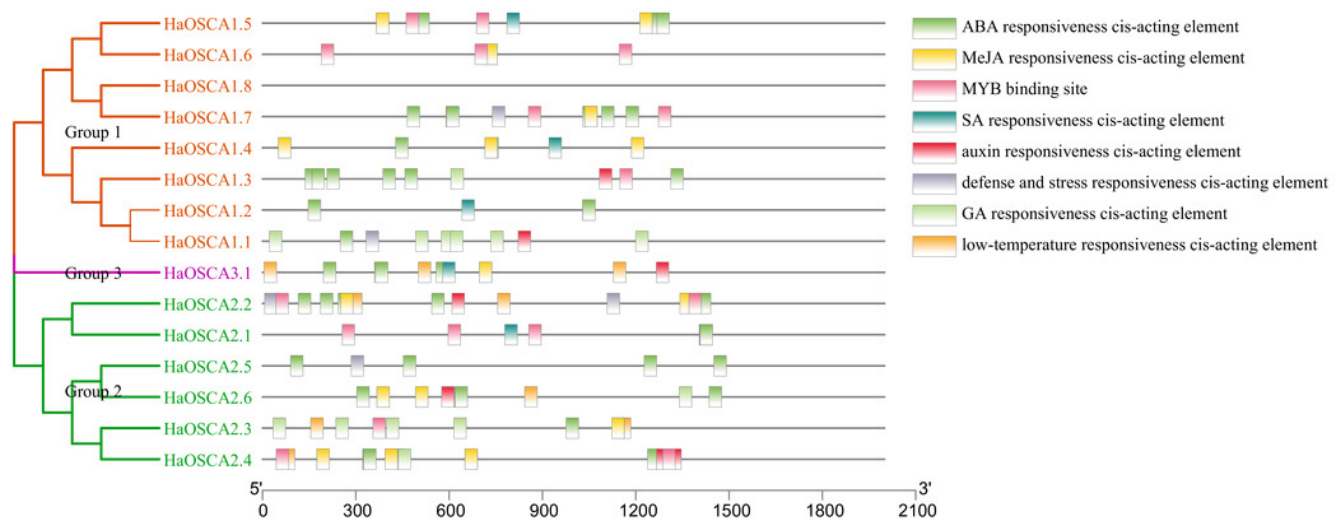
Figure 8

Fig. 8

Expression analysis of the sunflower OSCA gene. A) Heatmap of expression patterns in different tissues. B) Heatmap of expression patterns under different hormone treatments. C) Heatmap of expression patterns under salt stress.
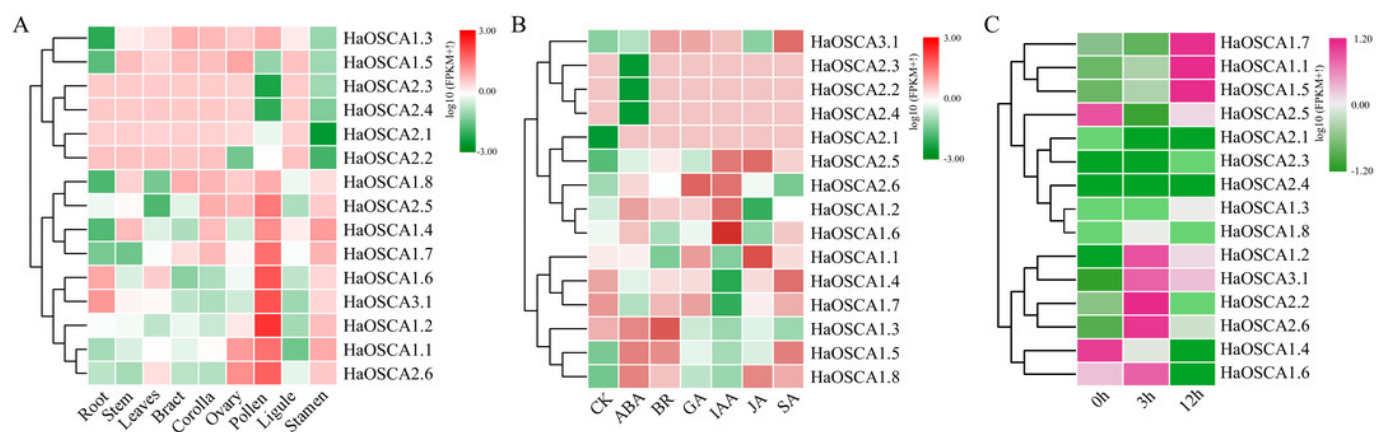
Figure 9

Fig. 9

Expression analysis of the sunflower OSCA gene under NaCl stress. Error bars represent the average of three replicates ± Sd. (*P<0.05; **P<0.01). The housekeeping gene *HaActin* was used as the internal reference gene.
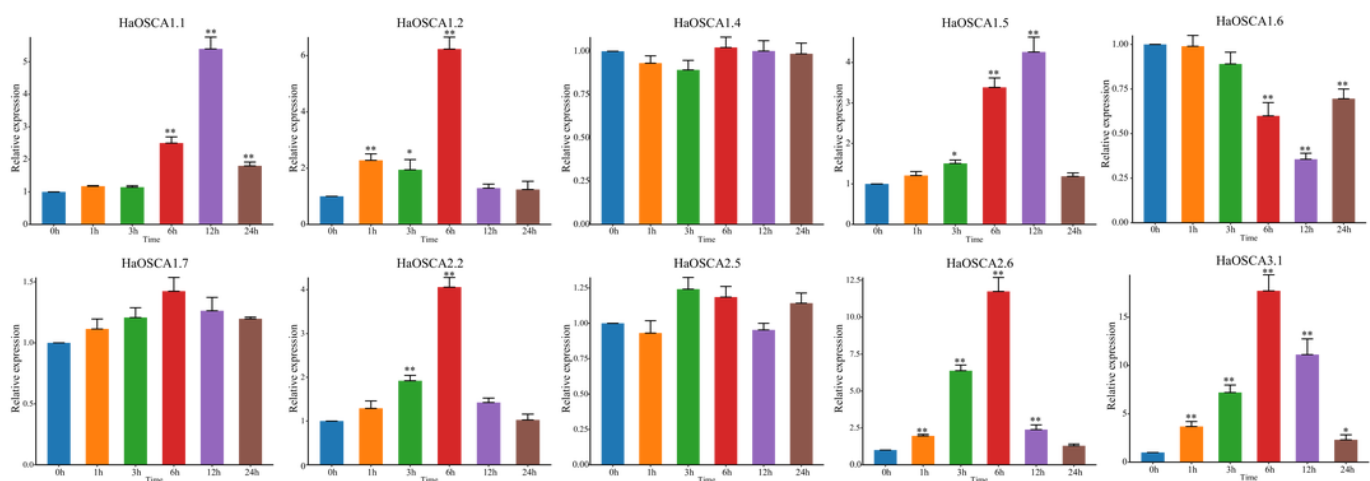
Figure 10

Fig. 10

A) Module partition of WGCNA clustering. B) sunflower OSCA coexpression network. C) GO enrichment analysis results of coexpression genes. D) KEGG pathway enrichment analysis results of coexpression genes.
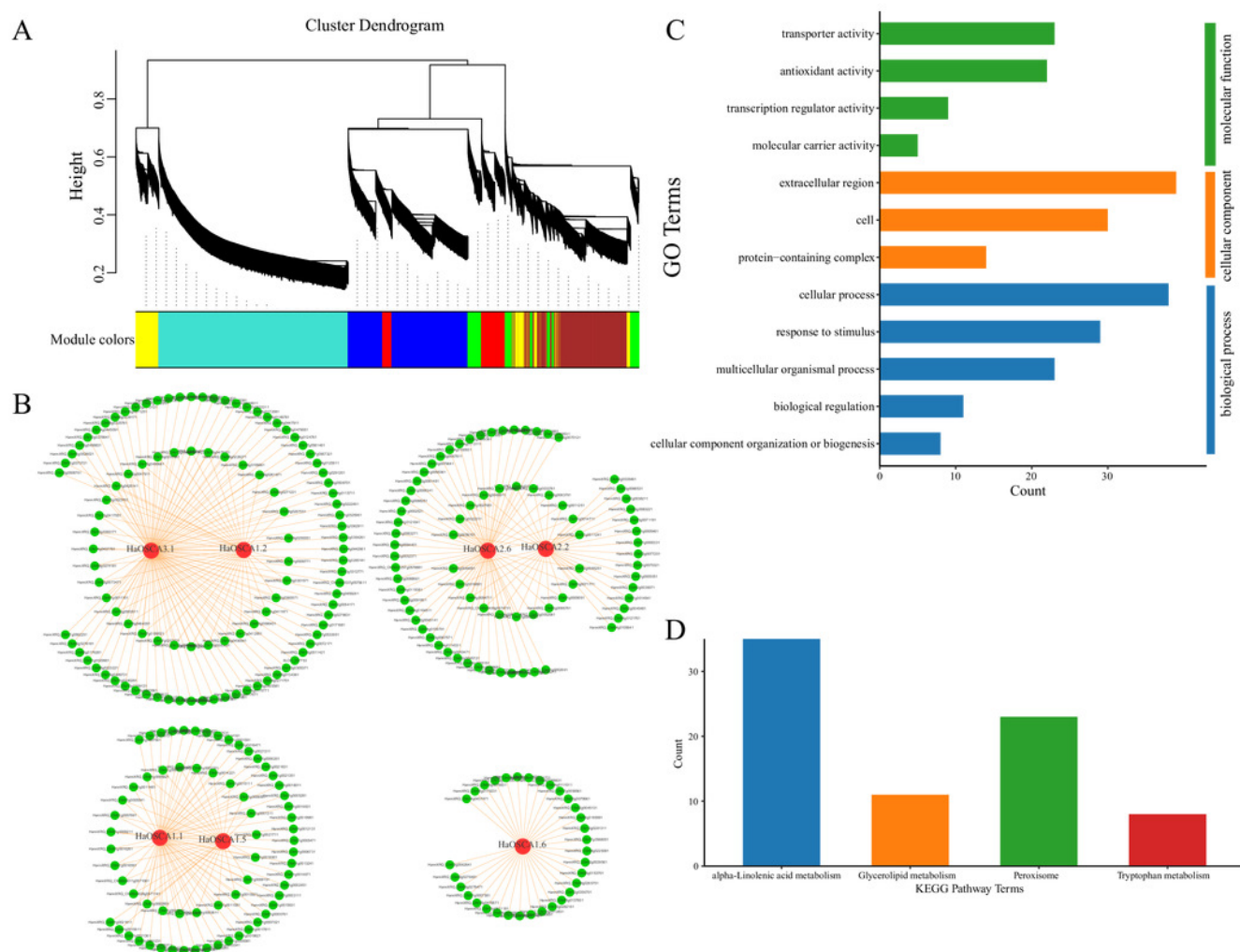
Table 1

Sunflower OSCA gene family member information.

1   Table 1. Sunflower OSCA gene family member information

Gene name	Gene ID	Open reading frame/bp	Protein length/aa	Relative molecular weight(r)/kDa	Theoretical isoelectric point (pI)	Subcellular localization
HaOSCA1.1	HannXRQ_Chr17g0558291	2097	698	79.86	8.07	Vacuole, Endoplasmic reticulum
HaOSCA1.2	HannXRQ_Chr16g0516941	2322	773	88.43	9.13	Endoplasmic reticulum
HaOSCA1.3	HannXRQ_Chr05g0129321	2274	757	86.85	9.73	Endoplasmic reticulum
HaOSCA1.4	HannXRQ_Chr16g0506581	2319	772	88.63	8.96	Endoplasmic reticulum
HaOSCA1.5	HannXRQ_Chr06g0164031	2310	769	87.77	9.36	Vacuole, Endoplasmic reticulum
HaOSCA1.6	HannXRQ_Chr04g0118011	2313	770	87.71	9.69	Vacuole, Endoplasmic reticulum
HaOSCA1.7	HannXRQ_Chr07g0194331	2340	779	89.00	9.94	Vacuole, Endoplasmic reticulum
HaOSCA1.8	HannXRQ_Chr03g0081691	2517	838	96.62	9.09	Chloroplast, Endoplasmic reticulum
HaOSCA2.1	HannXRQ_Chr17g0563981	1947	648	73.42	8.54	Chloroplast, Endoplasmic reticulum
HaOSCA2.2	HannXRQ_Chr16g0525101	1947	648	73.60	9.03	Chloroplast, Endoplasmic reticulum
HaOSCA2.3	HannXRQ_Chr07g0205641	2172	723	82.82	8.75	Golgi apparatus, Vacuole, Endoplasmic reticulum
HaOSCA2.4	HannXRQ_Chr07g0188761	2223	740	83.76	7.61	Chloroplast, Endoplasmic reticulum
HaOSCA2.5	HannXRQ_Chr09g0264291	2133	710	80.64	9.1	Golgi

						apparatus, Vacuole, Endoplasmic reticulum
HaOSCA2.6	HannXRQ_Chr12g0379201	2094	697	79.44	9.93	Mitochondrion, Vacuole, Endoplasmic reticulum
HaOSCA3.1	HannXRQ_Chr15g0481591	2145	714	80.99	9.29	Vacuole, Endoplasmic reticulum