Genomewide identification and analysis of the *OSCA* gene family in barley (*Hordeum vulgare* L.)

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Abstract. The hyperosmolality-gated calcium-permeable channels (*OSCA*) are considered to be osmotic sensors that play an important role in the early stages of hyperosmotic stress response. We analysed the physicochemical properties, chromosome distribution, phylogeny, gene structure and expression pattern of the *OSCA* gene family in barley, and investigated the expression of *HvOSCA* genes in barley under drought stress by quantitative real-time polymerase chain reaction (qRT-PCR). Finally, 14 members of the *OSCA* gene family were identified from barley, all containing the RSN1_7TM domain and often accompanied by the RSN1_TM and PHM7_cyt domains. According to the phylogenetic relationship, they were divided into four subgroups, and each with similar gene structures. Two types of duplicate events in the *HvOSCA* genes family evolved under the effect of purification selection. By analysing the *cis*-acting elements in the *HvOSCA* promoter, it was speculated that the barley *OSCA* gene family is involved in the regulation of multiple signalling pathways. The expression of *HvOSCA* genes in barley tissues and their relative expression under drought stress revealed that the gene family plays a role in plant organ differentiation, growth and development and coping with abiotic stress. These results provide new valuable information for the functional analysis of *HvOSCA* genes and the genetic improvement of barley, and provide an important reference for follow-up research.

Keywords. hyperosmolality-gated calcium-permeable channels; barley; bioinformatics; osmotic stress; expression analysis.

Introduction

Drought and salt stress are two major environmental factors that affect the distribution of plants in nature, limit the crop productivity and threaten food security (Zhu 2016). Previous studies have shown that plants respond to drought and salt stress mainly by regulating some potential signal transduction pathways and the expression of stress-responsive genes (Ingram and Bartels 1996; Zhu 2002, 2016; Bartels and Sunkar 2005; Munns 2005; Beno et al. 2016; Fang and Magwanga 2018). Since the changes in intracellular Ca$^{2+}$ in green algae first elucidated the function of Ca$^{2+}$ as a second messenger, transient increases in intracellular Ca$^{2+}$ concentration have been shown to be involved in many physiological processes, including the responses to abiotic stresses (Kudla et al. 2010). Among them, the hyperosmolarity-induced increase in [Ca$^{2+}$]$_i$ occurred within 5s, which may be the earliest event detected after plant hyperosmotic treatment (Knight et al. 1997). Different Ca$^{2+}$ signals encode different plant responses, such as CaM, CMLs, CDPKs, CBLs and CIPKs, which are encoded by different gene families that form complex signalling networks in plants, allowing flexible and accurate information processing (Batistic and Kudla 2012). Therefore, changes in calcium concentration are an important part of the signalling network of plant...
cells in response to the external environment and stimuli (Sanders et al. 2002; Hetherington and Brownlee 2004; Hepler 2005; Lecourieux et al. 2006). The study of calcium-related channels is of great significance for studying the changes of calcium and elucidating the responses of plants to various osmotic stresses.

The hyperosmolality-gated calcium-permeable channels (OSCA) family in Arabidopsis thaliana is considered to be hyperosmotic calcium-gated calcium permeation channels, which belong to calcium-permeable cation channel proteins in response to hyperosmotic stress, located on the plasma membrane and play a key role in the initial phase of the hyperosmotic stress response (Liu et al. 2018). Previous studies have shown that the OSCA gene family contains a highly conserved DUF221 domain, which includes seven transmembrane regions and was later renamed RSN1_7TM (Camargo et al. 2007; Liu et al. 2009; Rai et al. 2012). It is worth mentioning that the RSN1_TM and PHM7_cyt domains frequently appear near the RSN1_7TM domain. Currently, 11, 12, 16, and 21 OSCA genes have been identified in A. thaliana, rice, maize, Pyrus bretschneideri, and soybean, respectively (Li et al. 2015). In A. thaliana, mutants with increased calcium ions after osmotic induction were obtained by an unbiased forward genetic screening based on calcium imaging. The validated mutant was reduced hyperosmolality-induced [Ca$^{2+}$]i increase (oscal) (Yuan et al. 2014). At the same time, a gene that causes intracellular calcium influx during stimulation was also identified, named OSCAI.2 (Hou et al. 2014). In rice, the RSN1_7TM domain acts as an osmotic-sensing calcium channel, and OsOSCA3.1 has been identified as a gene involved in early drought response (Yuan et al. 2014; Li et al. 2015). Further studies found that OsOSCA1.4, AtOSCA1.8 and AtOSCA1.4 directly affect wheat yield, grain number per spike, apical fertility and other related plant traits. In soybean, 13 GmOSCA genes were directly affected by alkali stress and drought response. It can be seen that OSCA gene family members are closely related to plant growth and development, and osmotic stress response. Therefore, the study of OSCA genes will provide a theoretical basis for further exploring the mechanism of plant osmotic stress. At present, the OSCA gene family has been systematically identified and analysed in many species, but the genomewide identification and analysis of the OSCA gene family in barley has not been reported.

Barley has been extensively studied in genetics, genomics and breeding (Sreenivasulu et al. 2008). More and more researchers have systematically studied barley from the perspective of gene families, such as Hsp20 (Li and Liu 2019), HD-ZIP (Li et al. 2019), NBS (Habachi et al. 2018), GRAS (To et al. 2020), which provide us the opportunity to systematically study the OSCA gene family in barley. In this study, 14 HvOSCA genes were identified in barley using bioinformatics methods, and their physicochemical properties, phylogeny, gene structure, expression profiles in different tissues and developmental stages, and expression characteristics under abiotic stress were systematically analysed. This will provide a theoretical basis for further study on the function of HvOSCA gene family.

**Materials and methods**

**Identification and chromosomal localization of OSCA genes in barley**

The barley protein sequences were obtained from the Ensembl Plants database (http://plants.ensembl.org). The seed file of the conserved RSN1_7TM domain (Pfam: PF02714) was downloaded from the Pfam database (http://pfam.xfam.org/) (Finn et al. 2014). Hidden Markov model (HMM) of the conserved domain (RSN1_7TM) was established using HMMER software, and a preliminary study was carried out on it. All the retrieved sequences were submitted to Pfam and SMART (http://smart.embl-heidelberg.de/) to determine whether the candidate sequences contained the conserved RSN1_7TM domain.

The phytozome database (https://phytozome.jgi.doe.gov/) was searched for the chromosomal locations, CDS sequences and gene sequences of all barley OSCA genes. MapChart software (Voorrips 2002) was applied to map the chromosomal distribution of barley OSCA genes. Basic physical and chemical parameters were analysed with ProtParam (https://web.expasy.org/protparam/), including molecular weight (MW), isoelectric point (pI), grand average of hydropathicity (GRAVY), etc. Transmembrane regions (TMs) were predicted by TMHMM (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0).

**Phylogenetic and gene duplication analysis**

The protein sequences of the OSCA genes identified in A. thaliana, maize and rice were downloaded from the Uniprot database (https://www.uniprot.org/). The OSCA protein sequences of barley, A. thaliana, maize and rice were aligned using CLUSTALW (https://www.genome.jp/tools-bin/clustalw) using default parameters (Thompson et al. 1994; Larkin et al. 2007). The phylogenetic trees of 14 HvOSCAS, 11 ZmOSCAs, 15 AtOSCAs and 11 OsOSCAs were constructed based on the neighbour-joining method of MEGA-X software (Kumar et al. 2018) with default parameters.

Genome annotation files of different barley varieties were downloaded from Ensembi Plants and the Basic Circos tool in TBtools software (Chen et al. 2020) was used to visualize duplications among all OSCA genes on barley chromosomes. To elucidate the evolutionary selection of the OSCA gene family in barley, the synonymous
and nonsynonymous substitution rates between duplicated gene pairs were calculated using DanSP6 (Rozas et al. 2017).

**Protein conserved motif and gene structure analysis**

Visualization of conserved domains was performed using TBtools software. The online MEME tool (http://meme-suite.org/tools/meme) was used to identify and analyse the conserved motifs of OSCA proteins in barley. The Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/) was used to analyse the intron–exon pattern (Hu et al. 2015). The cis-acting elements of the promoters were analysed using plantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

**Gene expression analysis of HvOSCA genes**

Expression data for barley RNA-seq (ID: E-MTAB-2809) were downloaded from Expression Atlas (https://www.ebi.ac.uk/gxa/home) (Mayer et al. 2012) to determine the expression pattern of HvOSCA in eight different tissues or developmental stages (table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/). The cis-acting elements of the promoters were analysed using plantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

**RNA extraction and qRT-PCR**

Total RNA was extracted from barley samples by TRIZOL reagent (Invitrogen, USA) and treated with DNase I according to product instructions to remove genomic DNA contamination. Total RNA was reverse transcribed to generate cDNA using the Evo M-MLV RT kit with gDNA Clean for qPCR kit (Accurate Biotechnology, Hunan, China). The qRT-PCR analysis was performed using the SYBR Green Premix Pro Taq HS qPCR kit (Accurate Biotechnology, Hunan, China) on the CFX Connect Real-Time PCR System (BiO-RAD, USA). Specific primers for qRT-PCR were designed by Primer Express software and sent to company for synthesis (Sangon Biotech, Shanghai, China) (table 2 in electronic supplementary material). The HvActin gene was used as the internal control. Relative expression levels of OSCA genes in barley were calculated using the 2^{-ΔΔCT} method (Livak and Schmittgen 2001). Statistical comparative analysis of relative expression levels was performed using SPSS software.

**Plant materials, growth conditions and PEG stress treatment**

The expression pattern of OSCA gene in barley under drought stress was investigated using the H. vulgare cultivar Morex as material. Barley seeds were germinated and grown in a 16-h day / 8-h night cycle at 23°C and 60% relative humidity, and the most uniformly germinated seeds were selected and transplanted into a 96-well hydroponic tank filled with 750 mL of water. The seedlings at the three-leaf stage were transferred to a solution containing 10% (w/w) PEG8000 for further cultivation, while the control seedlings were still cultivated in the aqueous solution. Tissue samples were collected from the entire above-ground part of the seedlings excluding the roots 24, 36 and 48 h after the start of the drought stress treatment. After sampling was repeated thrice, they were immediately frozen in liquid nitrogen and then stored at −80°C for further extraction of total RNA.

**Table 1. Detailed information for 14 HvOSCA genes.**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene ID</th>
<th>Genomic location</th>
<th>Length (aa)</th>
<th>MW (kDa)</th>
<th>pI</th>
<th>GRAVY</th>
<th>TMs*</th>
<th>Subgroup</th>
</tr>
</thead>
<tbody>
<tr>
<td>HvOSCA01</td>
<td>HORVU1Hr1G004230</td>
<td>chr1H:8929487-8937936+</td>
<td>764</td>
<td>87.82</td>
<td>9.04</td>
<td>0.14</td>
<td>8</td>
<td>I</td>
</tr>
<tr>
<td>HvOSCA02</td>
<td>HORVU1Hr1G007290</td>
<td>chr1H:14277312-14283089+</td>
<td>592</td>
<td>68.05</td>
<td>9.01</td>
<td>0.05</td>
<td>5</td>
<td>I</td>
</tr>
<tr>
<td>HvOSCA03</td>
<td>HORVU3Hr1G045270</td>
<td>chr3H:296708818-296740420+</td>
<td>767</td>
<td>87.59</td>
<td>8.49</td>
<td>0.16</td>
<td>9</td>
<td>I</td>
</tr>
<tr>
<td>HvOSCA04</td>
<td>HORVU1Hr1G062630</td>
<td>chr1H:450961581-450968039+</td>
<td>767</td>
<td>87.58</td>
<td>6.70</td>
<td>0.14</td>
<td>8</td>
<td>I</td>
</tr>
<tr>
<td>HvOSCA05</td>
<td>HORVU1Hr1G051200</td>
<td>chr1H:379645200-379651249+</td>
<td>689</td>
<td>80.22</td>
<td>8.68</td>
<td>0.06</td>
<td>7</td>
<td>I</td>
</tr>
<tr>
<td>HvOSCA06</td>
<td>HORVU3Hr1G107240</td>
<td>chr3H:76800307-76807780+</td>
<td>725</td>
<td>81.49</td>
<td>8.94</td>
<td>0.15</td>
<td>9</td>
<td>II</td>
</tr>
<tr>
<td>HvOSCA07</td>
<td>HORVU5Hr1G018700</td>
<td>chr5H:672817167-672821507+</td>
<td>698</td>
<td>79.04</td>
<td>8.76</td>
<td>0.25</td>
<td>9</td>
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</tr>
<tr>
<td>HvOSCA08</td>
<td>HORVU4Hr1G009550</td>
<td>chr4H:27214849-27218985+</td>
<td>745</td>
<td>85.66</td>
<td>8.70</td>
<td>0.20</td>
<td>10</td>
<td>II</td>
</tr>
<tr>
<td>HvOSCA09</td>
<td>HORVU5Hr1G003420</td>
<td>chr4H:739986-7409174+</td>
<td>778</td>
<td>87.93</td>
<td>9.24</td>
<td>0.23</td>
<td>9</td>
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<tr>
<td>HvOSCA10</td>
<td>HORVU5Hr1G001660</td>
<td>chr5H:4723603-4727771+</td>
<td>781</td>
<td>88.57</td>
<td>9.20</td>
<td>0.20</td>
<td>9</td>
<td>II</td>
</tr>
<tr>
<td>HvOSCA11</td>
<td>HORVU5Hr1G001670</td>
<td>chr5H:4722940-4734718+</td>
<td>767</td>
<td>86.92</td>
<td>8.86</td>
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<td>II</td>
</tr>
<tr>
<td>HvOSCA12</td>
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<td>chr1H:420538033-420541659+</td>
<td>641</td>
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<td>9.09</td>
<td>0.23</td>
<td>9</td>
<td>III</td>
</tr>
<tr>
<td>HvOSCA13</td>
<td>HORVU2Hr1G060390</td>
<td>chr2H:403313393-403318326+</td>
<td>725</td>
<td>81.41</td>
<td>9.09</td>
<td>0.26</td>
<td>10</td>
<td>III</td>
</tr>
<tr>
<td>HvOSCA14</td>
<td>HORVU4Hr1G080450</td>
<td>chr4H:615697912-615705266+</td>
<td>833</td>
<td>94.16</td>
<td>8.98</td>
<td>0.12</td>
<td>9</td>
<td>IV</td>
</tr>
</tbody>
</table>

*TMs represents the number of transmembrane structures.
Identification and chromosome distribution of the OSCA genes in barley

To identify the OSCA proteins in barley, all the barley protein sequences were identified and screened with HMMER. After removing redundant and incomplete sequences, sequences with conserved RSN1_7TM domain were identified as candidate OSCA proteins, and then Pfam and SMART were used to further confirm the RSN1_7TM domain of candidate barley OSCA proteins. Finally, a total of 14 OSCA genes were identified in barley, named HvOSCA01–HvOSCA14. These 14 HvOSCA genes were located on five chromosomes and were unevenly distributed on each chromosome (figure 1). There were five HvOSCA genes on chromosome 1H, and three HvOSCA genes on chromosomes 4H and 5H, respectively. There were two HvOSCA genes on chromosome 3H and only one on chromosome 2H. No HvOSCA genes were found on both chromosomes 6H and 7H. It was worth mentioning that some HvOSCA genes were distributed in clusters, which was similar to the chromosomal distribution characteristics of many other gene families.

The physicochemical properties of the proteins encoded by the 14 HvOSCA genes were analysed (table 1; table 3 in electronic supplementary material). The length of the HvOSCA proteins ranged from 592 aa (HvOSCA02) to 833 aa (HvOSCA14), and their molecular weights (MWs) were within the scope of 68045.28 Da (HvOSCA02) to 94156.56 Da (HvOSCA14). The range of isoelectric points (pI) was 6.70–9.24. With the exception of HvOSCA04, the isoelectric points of the other members were significantly higher than 7.0, indicating that most of them were basic proteins, which was consistent with the results of the OSCA gene family of other species (Wang et al. 2019). The overall average of protein hydrophilicity value was calculated by dividing the sum of the hydrophilicity values of each residue by the total number of residues in the sequence, with positive and negative values reflecting hydrophobicity and hydrophilicity, respectively (Yang et al. 2019). Among the 14 HvOSCA, except for HvOSCA05, which was negative, the GRAVY scores of other HvOSCA proteins were all positive, indicating that most of the HvOSCA proteins were hydrophobic proteins. Transmembrane (TM) structure analysis showed that HvOSCA proteins contained 5–11 transmembrane structures, of which HvOSCA02 had the least transmembrane structures (only five). Compared with other species, the barley OSCA gene family had relatively a few transmembrane structures, and it was speculated that deletion events might have occurred (Li et al. 2015; Ganie et al. 2017).

Phylogenetic analysis of OSCA genes family

Phylogenetic analysis of HvOSCA genes revealed that the 14 HvOSCA genes were divided into four subgroups (figure 2;
Table 1. Subgroup I had five HvOSCAs (HvOSCA01–HvOSCA05), and subgroup II included HvOSCA06–HvOSCA11. Subgroup III had two members, HvOSCA12 and HvOSCA13, while subgroup IV had only one member (HvOSCA14). The evolutionary relationship between HvOSCAs showed that the number of HvOSCA genes in subgroups III and IV was significantly less than that in subgroups I and II. It was presumed that the variation events of gene members in subgroups III and IV were less than those in subgroups I and II.

To comprehensively analyse the phylogenetic relationship of OSCA genes, phylogenetic trees of OSCA gene family members in A. thaliana, rice, maize and barley were established using the above method. Likewise, all 51 OSCA genes were divided into four groups (figure 3). Subgroups I, II, III, and IV contained 21, 21, five, and four OSCA genes, respectively. Although subgroups III and IV contained relatively a few OSCA genes, they involve all four species. In addition, it can be seen from the evolutionary tree that the six pairs of orthologues genes with the nearest genetic relationship with barley (HvOSCA04/OsOSCA1.3, HvOSCA05/OsOSCA1.4, HvOSCA06/OsOSCA2.5, HvOSCA08/OsOSCA2.3, HvOSCA09/ZmOSCA2.2 and HvOSCA14/OsOSCA4.1) were all from rice and maize. Compared with the dicotyledonous plant A. thaliana, they were all monocotyledonous plants and always on adjacent branches, with a relatively close evolutionary relationship. This result was consistent with the existing consensus, indicating that the construction results of the evolutionary tree was relatively reliable.

Conserved domain and gene structure analyses of HvOSCA

By analysing the conserved domains of HvOSCAs, we found that HvOSCAs contain not only the most typical RSN1_7TM domain of the OSCA gene family, but also usually two other domains, RSN1_TM and PHM7_cyt, respectively (figure 4). The order of these three domains in the proteins was very regular. The RSN1_TM and RSN1_7TM domains were...
located at the N-terminal and C-terminal of all HvOSCA proteins, respectively, while PHM7_cyt domain was located in the middle, indicating that these three domains were relatively conserved in the HvOSCA gene family. Interestingly, among the 14 HvOSCAs, only HvOSCA02 lacked the RSN1_TM domain, which might help to investigate the function of the RSN1_TM domain in the future. The transmembrane structural features of the RSN1_7TM domain were elucidated and visualized by multiple sequence alignment (figure 5). The results showed that HvOSCA members contained 5–7 TMs in the RSN1_7TM region. These seven transmembrane domains were reported to be homologous to the calcium-activated chloride channel TMEM16 (Schroeder et al. 2008; Li et al. 2015). Among them, TM1–TM3 and TM6–TM7 were highly conserved among all HvOSCA genes, while TM4 and TM5 were not.

In this study, the conserved motifs of 14 HvOSCA proteins were analysed using MEME tool (figure 6). Among them, HvOSCA01 was taken as an example to illustrate the general distribution pattern of 20 motifs. Motifs 11, 10, 18 and 15 correspond to the RSN1_TM domain, motifs 4, 5, 20, 12, 1 and 8 correspond to the PHM7_cyt domain, and the rest correspond to the RSN1_7TM domain. The results showed that all HvOSCAs contained motifs 2, 3 and 8, indicating that this region is the most conserved region in the HvOSCA protein. In addition, some motifs are only present in specific subgroups. For example, motif 20 is present only in subgroup I, and motif 19 is present only in subgroup II, indicating functional differences between the different subgroups. Motif 12 was present in both subgroups I and III, suggesting that these two subgroups might have similar functions. Notably, HvOSCA14 had fewer motifs, which was consistent with the results of other species (Yin et al. 2021). Overall, the results of the conserved motif analysis were basically consistent with those of the phylogenetic analysis, i.e., the distribution patterns among members of the same subgroup were similar.

The results of intron–exon analysis showed that there were significant differences in the number of introns of the HvOSCA gene between different subgroups (figure 7). Subgroups I and II had 8–10 introns, subgroup III had 4–5
Figure 5. Multiple sequence alignment and transmembrane region prediction of RSN1_7TM conserved region in HvOSCA. The TMHMM online site is used to predict the transmembrane region. The regions between the black lines represent the RSN1_7TM conserved domain, and the blue lines represent the transmembrane region.
introns, and subgroup IV had no introns. The main mechanisms leading to this exon–intron structural difference are exon/intron acquisition/loss, exonization/pseudo-exonization and insertion/deletion, and the effects of each mechanism on structural differences are different (Xu et al. 2012). Moreover, the number of introns implies the potential ability of genes to form multiple spliceosomes. There was no significant difference in the length of each intron in these genes. In the other 13 gene sequences except HvOSCA14, the coding region corresponding to the RSN1_7TM domain was separated by 2–3 introns. The coding sequences of the other two domains, PHM7_cyt and RSN1_TM, were also separated by

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**Figure 6.** Conserved motif within OSCA gene family in barley. The data of motifs is obtained by MEME and then plotted using TBtools software. The 20 motifs are labelled with different colours.

**Figure 7.** Intron–exon distribution pattern of HvOSCA genes. The distribution pattern of intron–exon is identified by GSDS, in which purple represents the CDS region, orange represents the upstream and downstream, and the horizontal line is the intron. The green on the CDS is the PHM7_cyt domain, yellow represents the RSN1_7TM domain, and blue is RSN1_TM domain.
1–3 introns. It was interesting that the last exon encoding the PHM7_cyt domain was always located on the same exon as the first exon encoding the RSN1_7TM domain. In general, genes from adjacent branches tend to have similar gene structures during phylogeny.

**Gene duplication analysis of HvOSCA genes**

During the formation of gene families, gene duplication events are one of the main drivers of evolution, providing raw materials for the production of new genes. Among the various modes of duplication, segmental and tandem duplications are the two main causes of gene family expansion in plants (Moore and Purugganan 2003; Cannon et al. 2004; Kong et al. 2007). Among the 14 HvOSCA genes, we found that a pair of genes (HvOSCA10 and HvOSCA11) may be tandem duplication. HvOSCA10 and HvOSCA11 were located on the same chromosome, about 6 kb apart from each other, and then the Smith–Waterman algorithm showed that the two genes were more than 70% similar. Subsequently, segmental duplication events for a pair of genes HvOSCA09 and HvOSCA10 were identified. To further investigate the evolutionary pressure of HvOSCA genes, \( K_a/K_s \) values of duplicate gene pairs were calculated and analysed (table 4 in electronic supplementary material). The results showed that the \( K_a/K_s \) values of the above two pairs of genes were both less than 1.0. A \( K_a/K_s \) value of 1.0 indicates a neutral selection, a \( K_a/K_s \) value greater than 1.0 indicates positive selection, and a \( K_a/K_s \) value less than 1.0 indicates purified selection (Hurst 2002; Navarro and Barton 2003). Thus, purified selection had an impact on the evolution of barley OSCA gene family members.

**Collinearity analysis of OSCA genes**

Gene collinearity analysis reveals the functional and phylogenetic relationships of homologous genes among species (Yin et al. 2021). Therefore, this study visually analysed the collinear relationship between barley and six other species (figure 8), including three monocotyledons (sorghum, maize and rice) and three dicotyledons (A. thaliana, sunflower and soybean). The results showed that there were multiple OSCA gene collinear pairs between barley and the three monocotyledons, while there was only one pair between barley and dicotyledonous soybean, and even no OSCA gene collinear pair between barley, A. thaliana and sunflower. This suggests that the HvOSCA gene is more closely related in monocotyledons, which is consistent with the results of the above phylogenetic analysis and further validates our analysis. It was shown that the presence of OSCA gene collinear pairs in soybean may be associated with the large-scale amplification of the soybean genome (Yin et al. 2021). Interestingly, the occurrence of collinearity between barley and other plants was mainly concentrated in six genes, namely HvOSCA04, HvOSCA06, HvOSCA07, HvOSCA08, HvOSCA10 and HvOSCA13. After a long evolutionary process such as species differentiation, these genes still maintain a high degree of conservation and have the same or highly similar functions. From this, we can infer the function of the corresponding HvOSCA gene in barley based on the function of OSCA gene in other species.

**Analysis of cis-acting elements in the HvOSCA promoters**

To investigate the cis-acting elements of HvOSCA genes and their potential functions, we analysed the sequence of 2000 bp upstream of the start codon of HvOSCA genes (figure 9). The results showed that the type, number, and distribution of cis-acting elements in the HvOSCA genes were diverse, indicating that these genes may be different in function. All the HvOSCA genes contained TGACG-motif elements involved in the MeJA-response. The distribution of cis-acting elements differed in different subgroups. LTR elements associated with low temperature stress and ABRE elements involved in abscisic acid response were only existed in subgroup I, subgroup II and subgroup III, but not in subgroup IV. Research suggests that genes in different subgroups may make a collective response to stresses (Yin et al. 2021). Interestingly, the C-box element involved in light response is only present in the HvOSCA01 gene. In general, all HvOSCA genes contained at least two homeopathic elements, indicating that this gene family was involved in regulating a variety of signalling pathways.

**Expression profile of HvOSCA genes in different developmental stages and tissues of barley**

Gene expression profiles are usually related to the function of genes. In the present study, the expression profiles of HvOSCA genes in eight different tissues were analysed (figure 10; table 1 in electronic supplementary material). The results showed that there were significant differences in the expression of HvOSCA genes in different tissues or stages, indicating that the expression of HvOSCA gene is tissue-specific. HvOSCA03 was highly expressed in almost all developmental stages, suggesting that it may play an important role in the growth and development of eight tissues of barley. The expression of HvOSCA05 increased abruptly during caryopsis (5 dpa), which may be related to the formation of caryopsis. During the development of caryopsis from 5 dpa to 15 dpa, the expression of HvOSCA09 was upregulated, while the expression levels of HvOSCA03 and HvOSCA13 were downregulated, suggesting that the expression of HvOSCA is spatiotemporally specific. HvOSCA01 and HvOSCA07 had the highest expression in shoot (seedling) and internode, respectively, suggesting that HvOSCA gene may play a role in organ differentiation of barley plants. In addition, some genes,
Figure 8. Collinearity analysis of *OSCA* genes. The red lines indicate the synteny gene pairs between the two species. (a) Collinearity analysis of *OSCA* genes between barley and maize. (b) Collinearity analysis of *OSCA* genes between barley and sorghum. (c) Collinearity analysis of *OSCA* genes between barley and rice. (d) Collinearity analysis of *OSCA* genes between barley and soybean.

Figure 9. The *cis*-acting element analysis of *HvOSCA* promoters. The five *cis*-acting elements are represented by rectangles of different colours. ABRE, *cis*-acting element involved in the abscisic acid responsiveness; C-box, *cis*-acting regulatory element involved in light responsiveness; CCAAT-box, MYBHv1 binding site; LTR, *cis*-acting element involved in low-temperature responsiveness; TGACG-motif, *cis*-acting regulatory element involved in the MeJA-responsiveness.
such as HvOCA04, HvOCA08 and HvOCA12, were expressed at lower levels or not even expressed in the eight tissues. It can be seen that the expression of different members of HvOSCA in the same tissue is very different, implying that the HvOSCA gene family had undergone functional differentiation during evolution.

Expression analysis of OSCA gene family in barley under drought stress

Previous studies have shown that the OSCA gene is functionally associated with osmotic stress response in A. thaliana (Yuan et al. 2014). It was hypothesized that this gene family might also play a similar role in barley. Therefore, barley seedlings were treated with drought stress in this study and the relative expression of HvOSCA genes was analysed by qRT-PCR (figure 11; table 5 in electronic supplementary material). The results showed that three types of HvOSCA gene expression were present in the treatment group compared with the control group. In the first case, the expression of some genes increased over time, such as HvOSCA03, HvOSCA05 and HvOSCA13. In the second case, the expression of some genes first decreased and then increased, such as HvOSCA06, HvOSCA07, HvOSCA08 and HvOSCA14. In the third case, gene expression was first upregulated and then downregulated, such as HvOSCA09. It can be seen that the drought stress did induce differential expression of HvOSCA genes, that is, many HvOSCA genes responded to osmotic stress induced by drought treatment.

Discussion

In the process of plant growth and development, various osmotic changes run through it. Not only do various endogenous osmotic changes occur, such as changes in the content of substances that ensure plant growth and development in different periods, but more importantly, plants are subjected to exogenous osmotic stresses brought about by various harsh environments (Braam 2005). During the long evolutionary process, in order to survive under various environmental conditions, plants have adopted different strategies to sense and respond to these stresses, and have evolved a series of complex signal transduction networks that can rapidly respond to stresses (Qiu et al. 2011; Li et al. 2015; Gu et al. 2018). In previous studies, OSCA1 was found to be an important part of the plant osmotic receptor mechanism in
Arabidopsis, thus defining the OSCA family as a novel type of calcium-permeable ion channel (Yuan et al. 2014), which laid the foundation for us to further explore the role of OSCA gene family in the molecular mechanism of plant osmotic stress.

A total of 14 barley OSCA genes (HvOSCA01–HvOSCA14) were identified based on the barley protein database and whole genome sequencing database. Currently, the OSCA gene family of other species contained 11–21 members, and the number of HvOSCA members identified in this study was also within this range. The results of the phylogenetic analysis showed that the 14 HvOSCA genes could be divided into four subgroups, which is consistent with the evolutionary analysis of other species (Li et al. 2015; Gu et al. 2018; Ding et al. 2019). Phylogenetic analysis of barley and three other species showed that each subgroup contained OSCA genes from four species, and OSCA genes in monocotyledons and dicotyledons were distributed in each subgroup. Based on this, we speculated that the four subgroups of the OSCA gene family may have existed before the differentiation of these four species, even before the differentiation of monocotyledons and dicotyledons.

Generally speaking, gene duplication events play an active role in the evolution and expansion of the entire gene family, but the extent of the role varies in each species. This study found that there was a pair of tandem repeat genes in the HvOSCA gene family, namely HvOSCA10 and HvOSCA11. With regard to the fate of duplicated genes in the process of evolution, existing views suggested three scenarios. In the first case, one copy inherited the function of the ancestral gene and the other copy became a pseudogene. In the second case, one copy still retained the original function of the ancestral gene, while the other copy evolved a new function. The third case was the sub-functionalization of both copies, which shared the function of the ancestral genes (Moore and Purugganan 2005; Ma et al. 2014). Since the HvOSCA10 and HvOSCA11 genes were highly similar in sequence, gene structure, physicochemical properties and gene expression, and were located on adjacent branches of the evolutionary tree, it was speculated that they were more inclined to the third theory of evolution. Further analysis showed that the evolution of duplicated genes in the HvOSCA gene family underwent purifying selection. Purification selection would limit gene differentiation, resulting in duplicated genes that may retain some similar functions in the process of evolution (Yin et al. 2021), which also confirmed the rationality of our previous speculation.

The expression of the 14 HvOSCA genes varied greatly in different tissues and under different stress treatments. Previous studies have shown that the OSCA gene family is highly expressed in the roots of some plants. However, through expression profiling of members of the barley OSCA gene family, it was found that their expression in root tissue was not particularly high. It was speculated that the differences in their expression levels may be caused by different root stages. Although previous studies had reported the relationship between OSCA proteins and stress (Zhao et al. 2015), at present, the expression pattern of the OSCA gene family in barley stress response remains unclear. In this study, the expression patterns of HvOSCA genes under drought stress were divided into three cases. The results showed that many members of the HvOSCA gene family were involved in the response to drought stress. In previous studies, the relative expression of OsOSCA1.1, ZmOSCA1.1 and ZmOSCA1.4 genes were found to be upregulated after PEG treatment. Interestingly, in the present study, the HvOSCA03 and HvOSCA05 genes, which were homologous to the above genes, were also upregulated after drought stress, suggesting that these genes may have relatively conserved functions. This provided a clue for further investigation of the physiological function of HvOSCA as an osmotic sensor in barley.
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