Expression analysis of three SERK-like genes in barley under abiotic and biotic stresses

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ABSTRACT
Somatic embryogenesis receptor-like kinases (SERKs), a subfamily of receptor-like kinases, showed important roles in plant response to abiotic and biotic stresses in addition to embryogenesis and organogenesis in numerous plant species. In the present study, three orthologs of SERK genes (HvSERK1/2/3) were isolated from barley, and their expression patterns during in vitro culture of microspores as well as their responses to different stresses including salinity and powdery mildew were characterized. Sequence analysis suggested that three HvSERK genes were highly conserved in the grass family. Subcellular localization showed the HvSERK1 protein located on the plasma membrane. The HvSERK1 transcript was up-regulated during the microspore culture period, suggesting its roles in microspore embryogenesis. HvSERK1 and HvSERK3 showed the highest expression level in the leaves; however no difference was detected for HvSERK2 expression in different plants’ tissues. Under salt stress, all three HvSERK genes were quickly induced in microspore-derived embryogenic calli, whereas only HvSERK1 was up-regulated in the roots of barley seedlings. Moreover, only HvSERK2 was induced in the barley leaves upon powdery mildew inoculation. These results suggest that barley SERK genes may participate in barley microspores’ development and plant response toward salt and fungal stress, and the function of them has some evolutionary changes.

Introduction

To cope with various extracellular stresses, plants have developed various strategies which are usually based on three steps: recognition of the stress condition, signal transduction and regulation of gene expression leading to adaptive or protective physiological responses (Rathinasabapathi 2000; Denby & Gehring 2005; Valliyodan & Nguyen 2006). Receptor-like protein kinases (RLKs) are a large group of transmembrane proteins crucial for cell-to-cell and cell-to-environment communications, which process the cell signaling by three typical domain structures: an extracellular domain receiving environmental signals, a single-pass transmembrane domain anchoring the plasma membrane and a cytoplasmic kinase domain transducing signals to intracellular processes (Li 2010). The somatic embryogenesis receptor-like kinases (SERKs) belong to the leucine-rich repeat receptor-like kinase II group (LRR-RLKII) of receptor-like kinases, and the typical feature distinguishing them from other LRR-RLKII members is the presence of proline-rich SPP motif between the LRRs and the transmembrane domain (Hecht et al. 2001).

Previous studies showed that the SERKs are primarily involved in the somatic embryogenesis in both monocots and dicots, which express transiently and then decline afterward at globular stages (Schmidt et al. 1997; Somleva et al. 2000; Nolan et al. 2003; Singla et al. 2008; Li 2010). Therefore, SERKs are thought to be a molecular marker for somatic embryogenesis. Recent studies have explored that many SERK genes were implicated in plant response to biotic and abiotic stimuli (Santos & Aragão 2009). For instance, MsSERK1 can be induced by NAA (Nolan et al. 2003), whereas OsSERK1 is found to be activated by the blast fungus (Hu et al. 2005). Similarly, most of SERK or SERL genes in rice can be induced by brassino steroids (BR) and a few of them can also be triggered by 2, 4-D (Singla et al. 2009). These findings suggest that some evolutionary changes in SERK genes function in different species.

SERKs have been identified as well as characterized in numerous plant species, including model plant Arabidopsis (Hecht et al. 2001) and crops such as maize (Baudino et al. 2001), soybean (Yang et al. 2011), wheat (Singla et al. 2008), rice (Singla et al. 2009) and cotton (Pandey & Chaudhary 2014). All these studies have provided profound knowledge to understand the role of SERKs in plant development and environmental responses. However, the family of SERKs in barley (Hordeum vulgare L.), the fourth most important cereal crop which is cultivated across wide range of environments around the globe, has not yet been characterized.

In this study, three SERK genes were identified and isolated in barley embryogenic callus derived from isolated microspore culture, and their expression patterns in different culture points of microspores and different tissues as well as their responses to salt stress and powdery mildew stress were...
investigated for a better understanding of SERKs’ roles in barley development and environmental adaptation.

**Materials and methods**

**Plant materials and treatments**

Barley (*Hordeum vulgare* L.) cv. Hua-30, a popular variety cultivated in the area of Yangtze River delta of China, was grown in the farm of Shanghai Academy of Agricultural Sciences, Shanghai, China.

The microspores were isolated from the pretreated young spikes of barley Hua-30 and cultured *in vitro* following the procedures described by Lu et al. (2008) to induce MDEC (microspore derived embryonic callus). After 19-day culture, the induction medium was changed with addition of 8.36 mM·L⁻¹ NaCl as salt stress followed by Liu et al. (2016), and the MDECs were collected at 0, 1, 2, 4, 6, 12 and 24 hours after the abiotic treatment for RNA isolation.

The seeds of barley Hua-30 were germinated on wet filter paper, and the seedlings were grown hydroponically in a modified Hoagland’s solution described by Tavakkoli et al. (2012) at 20–22°C with a photoperiod of 12 h. When the seedlings were at the two-leaf stage, we separated the leaves, stem and roots from the seedlings for RNA isolation. To investigate the SERK genes’ expression in abiotic stress, Hoagland’s solution was changed with addition of 300 mM NaCl as salt stress (Seckin et al., 2010), and the barley roots and leaves were collected at 0, 1, 2, 4, 6, 12 and 24 hours after the abiotic treatment for RNA isolation. To induce biotic stress, the seedlings were inoculated with mixed races of *Blumeria graminis* f. sp. hordei (*Bgh*), and the leaves were sampled at 0, 1, 2, 4, 6, 12 and 24 hours after the biotic stress treatment for RNA isolation.

To clone the full length of cDNAs for all SERK genes in barley cv. Hua-30, the MDECs produced at 19d culture were used for RNA extraction. For analysis of the gene expression pattern in specific tissues, the MDECs produced at 3d, 7d, 9d and 21d culture and the tissues (leaf, stem and root) of seedlings at two-leaf stage without any stress were used for RNA extraction. Three biological replicates were prepared for each assay.

**Identification of SERK gene family in barley**

To identify SERK homologs in barley, the amino acid sequences of reported SERK proteins in model plants including *Arabidopsis* and rice were used to search in the International Barley Sequencing Consortium (http://webblast.ipk-gatersleben.de/barley), and the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/BLAST/nr/EST) using the TBLASTN program with a cut-off E-value of 1×10⁻¹⁰. Nine sequences were retrieved, downloaded and used to remove redundant sequences following multiple alignment by ClustalX (version 1.83) program, among which three SERK genes were identified in barley by typical SERK characters. As the available sequences of three SERK genes were from high throughput sequencing in barley cv. Morex, the ORF of three SERK candidates was homolog cloned for verification from barley cultivar Hua-30 by PCR amplification with cDNA of microspore-derived embryogenic callus as template using specific primers.

**In silico analysis**

The BLAST tool (http://www.ncbi.nlm.nih.gov/blast/) was used to analyze the conserved motifs and domain structure of SERK while full open reading frame (ORF) was predicted by online available ORF finder software (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The retrieved sequences were aligned using the ClustalX 1.83 program and phylogenetic associations were inferred using the neighbor-joining method in MEGA 4.0 software.

**Total RNA extraction and quantitative reverse transcription-PCR (qRT-PCR)**

Total RNA of all samples collected in this study was extracted by Trizol (Invitrogen, Carlsbad, CA, U.S.A.) following the instructions of the manufacturer. The cDNA was synthesized with oligo (dT)₂₀ primers by AMV reverse transcriptase (Life, U.S.A.). The quantitative reverse transcription-PCR (qRT-PCR) assay was performed using SYBR Green/Flourescein qPCR master mix (ToYoBo) in ABI step one system. The expression of barley actin gene was used as the internal control for each qRT-PCR. All reactions were conducted in three biological replicates and the reactions with non-template were used as negative controls. The comparative threshold 2⁻ΔΔCT method was applied to quantify the relative expression of the given gene (Livak & Schmittgen 2001). All the primers used in this study are listed in Table S1.

**Subcellular localization of HvSERK1**

*BglII* and *NotI* sites were added to the 5’ and 3’ ends of the full-length ORF of *HvSERK1*, respectively, with the stop codon deleted by appropriate design of the *HvSERK1*-NotI-R primer. The PCR product and the eGFP vector were cut by *BglII* and *NotI*, and the fragments were ligated to produce the fusion gene expression vector p35S::*HvSERK1*-GFP::Nos3. The recombinant constructs were verified and used for the subcellular localization. For protoplasts transforming, about 20 µL total plasmid DNA (1.5 µg/µL) were transformed into 200 µL barley mesophyll protoplasts prepared from 5- to 7-day-old Hua-30 seedlings. GFP signals were assessed by Fluorescence microscope (Olympus) at 16–20 h after transformation.

**Results and discussion**

**Identification of the barley SERK genes**

Nine homologs of barley SERK genes were obtained from the barley whole genomic sequencing data deposited in

**Table 1. Information of three barley SERK genes.**

| Name     | Accession number | ORF length | 5’ UTR | 3’ UTR | Mol wt KDa | pl | Chromosome Locus | Similarity between Hua30 and Morex |
|----------|------------------|------------|--------|--------|------------|----|-----------------|-----------------------------------|
| HvSERK1  | AK372118         | 1884       | 165    | 308    | 69.09      | 5.86| 2H              | 100%                              |
| HvSERK2  | AK252995         | 1887       | 280    | 257    | 68.85      | 5.42| 6H              | 100%                              |
| HvSERK3  | AK374641         | 1869       | 103    | 272    | 68.49      | 5.8 | 7H              | 100%                              |
IPK database using the TBlastN program against other known SERK proteins. Three of them were identified as SERK genes according to the presence of SPP motif between the LRRs and the transmembrane domain, which shares higher identity to the specific motif of *AtSERK1/2/3* as compared to the remaining six SERL genes. To verify the full-

Figure 1. Multiple sequence alignment of predicted amino acid sequence showing sequence similarity of three SERK proteins from barley with reported SERK sequences. Characteristic domains of three barley SERK proteins are indicated (signal peptide, leucine zipper, LRR1 to LRR5 leucine-rich repeats 1–5, SPP serine proline proline, transmembrane domain, kinase domain, C-terminal). OsSERK1 (BAD05545.1) and OsSERK2 (XP_015636497.1) from Oryza sativa, ZmSERK1 (NP_001105132.1) from Zea mays, AtSERK1 (NP_177328.1) and AtSERK2 (AAK68073.1) from Arabidopsis thaliana.
length cDNA sequences of three SERK genes available by high throughput sequencing in barley cv. Morex (19), three SERK genes were cloned from barley cultivar Hua-30 microspore-derived embryogenic callus, which were then designated as HvSERK1, HvSERK2 and HvSERK3, respectively. It was found that the ORF sequences of each SERK gene from Hua-30 were same as those from Morex (Table 1). The amino acid sequences of three SERK genes were aligned with five other reported SERK proteins from monocots and dicots which indicated that these all proteins shared typical characteristic conserved domains of the SERK, including five LRRs, a SPP motif, TM and kinase domains (Figure 1). Multiple sequence alignments of deduced amino acid indicated that HvSERK1/SERK2/SERK3 showed a high level of sequence and protein domain homology with SERK proteins from other species. These suggested that the SERKs in grass family remained fairly conserved during the evolution not only in terms of sequence but also for number of domains and domain structure.

A phylogenetic tree was constructed used SERK homologs from different species, such as rice, Arabidopsis, Triticum aestivum, Zea mays, Setaria italica and Sorghum bicolor. The HvSERK1 and HvSERK2 were close to the TaSERK1 and TaSERK2, respectively, while HvSERK3 has a homology close to that of OsSERK1 (Figure 2). Moreover clustering of all the three SERKs with the homologs from grass species indicated that SERK genes remained conserved during evolution of monocot species.

Expression of three SERK genes during the microspore culture and in different tissues

qRT-PCR analysis showed that the expression of HvSERK1 was up-regulated from three days of cultured microspores, and maintained the high expression at the following time points. HvSERK3 and HvSERK3’s expression did not have a significant change at all culture points (Figures 3 and 4(A)). Tissue-specific expression was analyzed for three SERK genes in barley. All the three SERK genes’ expression could be detected in the roots, stems and leaves from two-leaf barley seedlings. For HvSERK1 and HvSERK3, the highest expression level was detected in leaves (Figure 4(B)). However, the expression of HvSERK2 showed no significant difference in all the detected tissues.

Lot of evidences showed that SERKs play an important role in plant development. The sunflower SERK gene expression was correlated to induction of two different developmental pathways, somatic embryogenesis and shoot organogenesis (Thomas et al. 2004). In Arabidopsis, AtSERK1 and AtSERK2 genes are essential in male sporogenesis (Albrecht et al. 2005). In accordance with this our results suggested that HvSERK1 participates in barley microspore embryogenesis. And the highest expression of HvSERK1 and HvSERK3 in young leaves suggested that both of them have a role in barley leaves. The lack of difference for HvSERK2 expression in all the tissues indicated it was a basal gene in the barley tissues.

Subcellular localization of HvSERK1

As the three genes have similar domains, we used HvSERK1 as an example to check the subcellular localization of HvSERKs. The fusion protein HvSERK1-GFP was introduced in barley protoplast. The result showed that green fluorescence was mainly localized in the plasma membrane, whereas in the case of control treatment GFP signals were distributed evenly in the cell compartment including the plasma membrane, cytoplasm and nucleus (Figure 5). This result was consistent with the sequence analysis of HvSERK1 which depicted the presence of the transmembrane domain and predicted the protein localization on the plasma membrane. Given the high homology in domain structures and presence of transmembrane domain between three SERK proteins, it was assumed that HvSERK2 and HvSERK3 would also be located on the plasma membrane.

The barley SERK genes expression pattern under salinity stress

To investigate the role of three barley SERK genes under salt stress, the expressions in different tissues were examined using 19-day MDEC (8.56 mM.L⁻¹ NaCl) as well as in
roots from two-leaf seedlings (300 mM NaCl). In the MDEC treated with NaCl, all three \textit{SERK} genes were quickly induced at 1 hour post treatment (hpt), especially the expression of \textit{HvSERK3} up-regulated (10–100 folds) at the following time points after treatment (Figure 6(A)). It indicated that all the three \textit{HvSERK} genes might participate in the salt stress responses in the MDEC and the response of \textit{HvSERK3} is more sensitive than that of the other two genes.

On other hand, when barely seedlings were subjected to salt stress, only \textit{HvSERK1} was up-regulated in the barley root at 12 hpt and remained at a high level until 24 hpt (Figure 6(B)). This indicated that \textit{HvSERK1} may participate positively in the salt tolerance pathway in the roots. The transcript level of the \textit{HvSERK} genes were also investigated in leaves after salt stress. The results showed that \textit{HvSERK1} was up-regulated at 12 hpt and remained at a high level from 12 hpt. While \textit{HvSERK3} was up-regulated at 12 hpt it reduced to normal level at 24 hpt. However, no significant variation was observed in the expression level of \textit{HvSERK2} at any time interval. These results indicated that \textit{HvSERK1} and \textit{HvSERK3} may participate in the NaCl tolerance pathway in leaves.

Lots of evidence showed the \textit{SERK} genes play roles in plant response to abiotic stimuli. In rice, \textit{OsSERK1} involved in stress tolerance are stimulated by ABA (Hu et al. 2005). Salinity stress can inhibit the germination and seedling growth of rice, and Brassinosteroids (BR) were found to reverse these inhibitory effects (Anuradha & Seeta Ram Rao 2001). The \textit{Arabidopsis} \textit{SERK1}, \textit{SERK3} and \textit{SERK4} have been reported to be involved in BR signaling (Albrecht et al. 2008). This indicated \textit{SERK} may play a role in salinity stress. The results showed \textit{HvSERK1} and \textit{HvSERK3} has response to the salt stimulate in barley, this suggested the two genes play role in the NaCl tolerance pathway of barley. And this needs further research on the two \textit{SERK} genes.

In contrast to this the \textit{SERK} genes in MDEC showed a different expression pattern from the seeding tissues, either regarding the response time or the expression abundance. The salt tolerance mechanisms involved at the whole plant level were different from those involved at the plant cell level (Adams et al. 1992). Due to few signal transduction among cells in response to different abiotic stresses, it was efficient to screen out the genes involved in stress responses in MDEC. Thus MDEC may provide a very good platform to search genes in the plant-abiotic interaction.

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The barley \textit{SERK} genes’ expression pattern after being infected by powdery mildew

\textit{SERK} was reported to play a positive role in the pathogen defense of plants (Li et al. 2002). To investigate whether the three \textit{SERK} were involved in the barley–pathogen interaction, we inoculated the leaves of barley seedlings with \textit{Bgh}. The qRT-PCR results showed that \textit{HvSERK2} was up-regulated at 12 hours post inoculation (hpi), then regained its normal level afterward; however the other two genes’ (\textit{HvSERK1}/\textit{HvSERK3}) expression did not change after \textit{Bgt} inoculation (Figure 7).
In rice, SERK gene OsSERK1 was induced by defense signaling molecules such as salicylic acid, jasmonic acid and abscisic acid, and was associated with host cell death in the resistance to rice blast fungus (Hu et al. 2005). The OsSERK2 can positively regulate the immunity mediated by XA21 and XA3 to the Xanthomonas oryzae pv. oryzae (Xoo) (Chen et al. 2014). It was shown that the Arabidopsis SERK3/BAK1 participated in the pathway of brassino steroid (BR) signal transduction to regulate the plant development and innate immunity (He et al. 2007; Heese et al. 2007; Clouse. 2011). In this work, we showed that among the three barley SERK genes, only HvSERK2 was induced by Bgh in the barley leaves. This suggested HvSERK2 may be involved in the barley resistance to Bgh; however further investigation is needed to get better understanding about the role of HvSERK2 against Bgh resistance.

**Conclusion**

In this study, three SERK genes HvSERK1, HvSERK2 and HvSERK3 have been identified in barley cultivar Hua-30. And three barley SERK genes comprise a multigene family.
Gene expression data also suggested that barley SERKs are involved in plant biotic or abiotic stress. And the three HvSERK genes (HvSERK1/2/3) have come up with different functions toward the same stress.

Disclosure statement

No potential conflict of interest was reported by the authors.

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