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Short Communication

Cloning and characterization of up-regulated *HbSINA4* gene induced by drought stress in Tibetan hulless barley

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ABSTRACT. Hulless barley is an important crop cereal in Tibetan, China. Drought is a major abiotic stress in barley production. In this study, we cloned the drought-related *HbSINA4* gene from the variety 'Himalaya 10' and analyzed its expression patterns under different drought and rehydration conditions. The cDNA of *HbSINA4* was 1052 bp long, including an open reading frame of 771 bp that encoded a protein of 256 amino acids. The molecular weight of HbSINA4 protein was predicted to be 29.53 kDa and the theoretical pI was 8.32. Bioinformatic analysis showed that the *HbSINA4* gene contained a protein kinase domain profile family signature motif, with high similarity to that of *Oryza sativa* and *Brachypodium distachyon*. Real-time polymerase chain reaction (PCR) assays revealed that gene

expression declined rapidly with increasing drought stress; in contrast, its expression increased after rehydration treatment. Therefore, the *HbSINA4* gene responds to the drought stress and plays an important role in barely drought resistance. Furthermore, our results provide information which may be useful in other temperate crop studies and in aiding resistance to drought.

Key words: Barley; Drought stress; Gene cloning; Gene expression; *HbSINA4*; *Hordeum vulgare*

INTRODUCTION

Because of increased global warming, drought stress has threatened the world's food security. Additionally, the world human population is predicted to reach 9.4 billion by 2050, as predicted by the UN, and more food will be required to feed this large number of people. Drought stress is one of the more important environmental stresses affecting agricultural productivity worldwide (Boyer, 1982). In crops, drought can affect vegetative growth and yield formation at any developmental stage depending on the cropping region. Severe drought stress can even cause plant death (Zhao and Li, 1999). To face these challenges, plant breeders and geneticists are seeking strategies to overcome or reduce the detrimental effects of drought. Drought-resistant varieties have been developed in many breeding programs. To achieve this, the first step is to understand the biological processes and genetic basis of plant responses to drought.

The drought resistance of crops is controlled by multiple genes, including those involved in signal transduction and transcriptional regulation, as well as the expression of genes involved in plant protection, defense, and resistance stresses (Wang et al., 2003). Drought resistance is closely related to a range of biological process, such as osmotic regulation, autoxidation, photosynthesis, plant hormones, and dehydration-protection protein metabolism, indicating that the response is complex (Wang, 2013). Numerous physiological and biochemical indices, methods, and technologies to combat drought stress have been developed in previous studies (Singh et al., 1972; Acar et al., 2001). Several genes were identified to be induced by drought (Diab et al., 2004; Guo et al., 2009; Zhang et al., 2009). Quantitative trait loci mapping has been used in *Arabidopsis thaliana*, *Triticum aestivum*, and *Oryza sativa*, among others (Zhao et al., 2005; Wang, 2007; Nie, 2009). Based on chlorophyll a fluorescence OLKJIP under drought stress and re-watering, Oukarroum et al. (2007) evaluated chlorophyll content and fluorescence parameters as indicators of drought tolerance in barley. Barley is one of the most important cereal crops in many countries, including China. In most countries, barley is often the only possible rain-fed crop that farmers can grow, and is often subjected to extreme water deficit during the dry season. In Tibet, the early stage of highland barley growth is typically threatened by drought. In order to improve the yield and quality of highland barley, it is crucial to select drought-resistant elite varieties, which can be developed by understanding the genetic mechanisms of drought resistance.

The seven in absentia (*SINA*) protein belongs to the ubiquitin ligase *E3* family. *SINA* protein plays important roles in many biological processes through ubiquitination of specific proteins. Little is known about the function of *SINA* family in plant, with a few exceptions. For example, *SINAT5* has been found to be related to lateral root formation in *A. thaliana* (Xie et al., 2002). Additionally, Welsch et al. (2007) reported that an interaction between *SINAT2*

and transcription factor *AtRAP2.2* are involved in the formation of carotene. Den Herder et al. (2008) found that *SINA* protein can affect plant growth and nodule formation. In this study, we cloned the *SINA* gene, which is related to resistance to drought, and then analyzed its expression patterns under drought stress.

MATERIAL AND METHODS

Plant materials and drought treatments

An elite hulless barley cultivar 'Himalaya 10', which shows good drought tolerance, was used. This cultivar was kindly provided by Tibetan Academy of Agriculture and Animal Husbandry Sciences. Soil used for growing barley was measured for water content, and the moisture was adjusted to 33.4% by adding water. Seedlings of Himalaya 10 were grown in a greenhouse under identical conditions at a temperature of 23°/15°C (day/night) and relative humidity of 10-20%. Prior to drought stress treatment, seedlings were watered every 2 days to maintain the relative soil moisture content (RSMC) at 33.4%. The drought stress began when the seedlings grew to 2-and-a-half leaf stage (18 days after sowing). Drought stress included 6 different levels by limiting the water supply, with RSMC values of 33.4, 27.5, 21.1, 15.5, 9.8, and 4.8%. At each RSMC level, leaf samples were harvested. After drought stress, the remaining seedlings at 4.8% RSMC were rewatered to restore the RSMC to 33.4%, and the leaf samples since rehydration (2, 4, and 8 h after RSMC 33.4%) were collected. All leaf samples were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction.

Cloning of cDNA of *HbSINA4*

Total RNA was extracted from the leaf samples and treated with DNase I. Primers were produced using Primer Premier 5.0 based on the sequences from the transcriptome sequencing analysis results (data not shown). Total RNA was reversely transcribed into cDNA. The primers used were A1-FP (AGGTACCATGTACCCTCCGATCCACCAG) and A1-RP (AACTAGTTCAGCTGAAGAGATTAGGGATGC). Real-time polymerase chain reaction (RT-PCR, S-1000 Thermal Cycler, Bio-Rad, Hercules, CA, USA) amplification was carried out in a 20-μL volume containing 0.2 μL 5 U/μL Taq DNA polymerase, 2 μL 10X PCR buffer, 1.6 μL 10 mM dNTP mixture, 0.8 μL 10 μM of each primer, and distilled water. After complete denaturation of the template DNA at 94°C for 4 min, PCR was performed with 34 cycles of 50 s at 94°C, 45 s at 51°C, and 1 min at 72°C, followed by final extension at 72°C for 10 min. PCR products were visualized on 1.5% agarose gel, purified using an ENZA™ gel extraction kit (Omega, Norcross, GA, USA), and then cloned into the pMD19-T vector (TaKaRa, Shiga, Japan) according to manufacturer instructions. All samples were sequenced by the Beijing Genomics Institute (Beijing, China).

Bioinformatics analysis of *HbSINA4*

Databases such as NCBI (http://ncbi.nlm.nih.gov/), ExPAsy (http://www.expasy.org/tools/pitool.html), TMHMM (http://www.cbs.dtu.dk/services/TMHMM/), TargeP (http://www.cbs.dtu.dk/services/SignalP), and PSORT (http://psort.hgc.jp/form.html), were used to analyze the sequences of *HbSINA4*. Based on the predicted *HbSINA4* amino acid sequence,

the package MEGA5.1 was used for amino acid sequence homology comparison analysis and to construct the evolutionary tree.

RT-PCR analysis of *HbSINA4*

RT-PCR was conducted using the SYBR Premix Ex TaqTM Kit, and the quantitative primers [A2-FP (TGGGTGACATCAGGTGGTGTTTGG) and A2-RP (GAGCATTCAGATC CCGCATAA)] were also designed according to the *HbSINA4* sequence. β-Tublin was used as a reference gene [A3-FP (CCAAGTTCTGGGAGGTGATCTG) and A3-RP (TTGTAGTAGA CGTTGATGCGCTC)]. RT-PCR data was analyzed using the double standard curve method as described by Zhang et al. (2005).

RESULTS

HbSINA4 cloning and bioinformatics analysis

A cDNA of 1052 bp was amplified successfully (Figure 1). Using the ORF finder procedure in NCBI, the 5'- and 3'-untranslated regions were 82 and 1082 bp in length, respectively. The open reading frame of this gene was 771 bp, coding for 256 amino acids (Figure 2). The predicted molecular weight of this polypeptide was 29.53 kDa and the isoelectric point was 8.32. Prosite Scan analysis indicated that this polypeptide included the family signature motifs of zinc finger SIAH-type profile (single underline in Figure 2), 6 protein kinase C phosphorylation sites (bold in Figure 2), 4 N-nutmeg acylation sites (wave underline in Figure 2), 2 N-glycosylation sites (italics in Figure 2), amidation site (double underline in Figure 2), and 2 casein kinase II phosphorylation sites (dotted underline in Figure 2). The TMHMM database revealed that this protein did have the membrane transfer function. Signal IP3.0 analysis revealed that this protein did not have a signal peptide, indicating that it is a non-secretory protein. PSORT subcellular localization analysis showed that this protein was located in the cytoplasm.

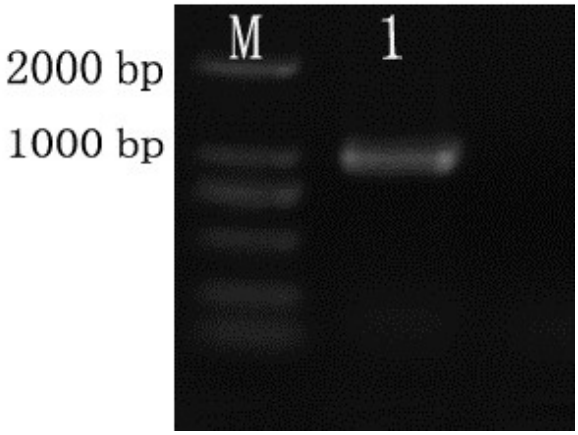

Figure 1. Amplification of the *HbSINA4* gene from Tibetan hulless barley. *Lane M* = DL2000; *lane 1 = HbSINA4*.

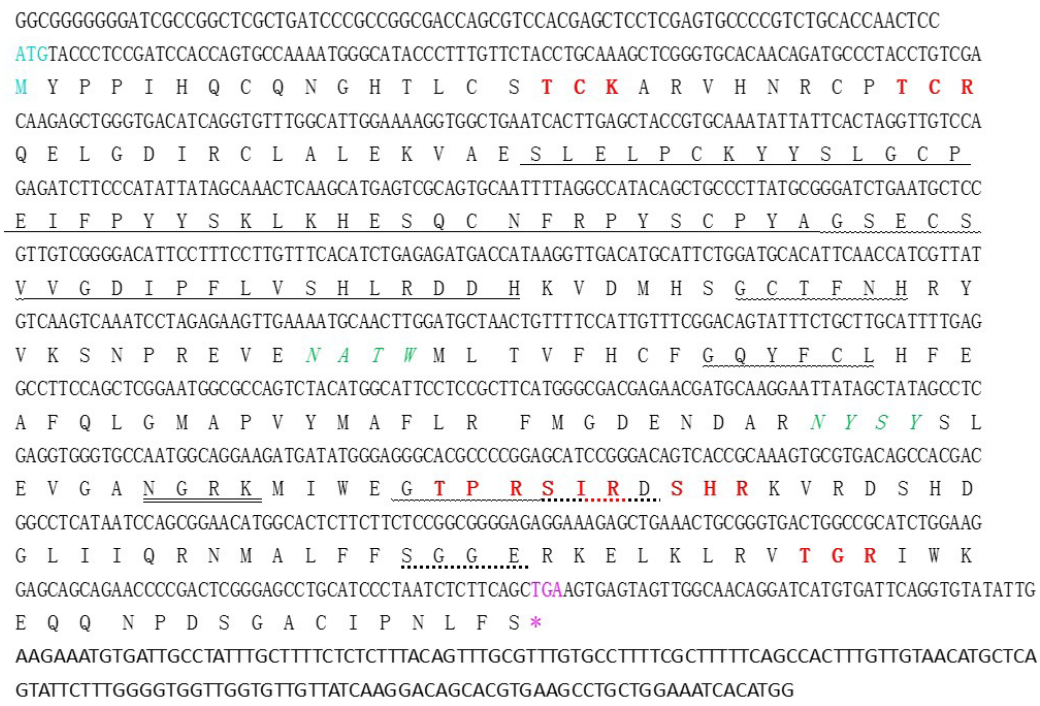

Figure 2. Nucleotide and predicted amino acid sequences of the *HbSINA4* gene (single underline: zinc finger SIAH-type profile family signature residuals; bold: proteinase C phosphorate site; wavy line: N-myristoylation site; italic: N-glycosylation site; double line: amidation site; dashed line: casein kinase II phosphorylation sites).

BLASTn analysis indicated that this sequence of *SINA* had high similarity (90-96%) to *SINA* found in *O. sativa*, millet, maize, *Brachypodium distachyon*, *Solamum lycopersicum*, beans, *Solanum tuberosum*, and string beans. Interfering amino acid sequence homology, analysis (Figure 3) suggested a similarity of 92-98% between *HbSINA4* and the orthologs in these species. Based on the amino acid sequence, we built a phylogenetic tree, which revealed consistent evolution between *HbSINA4* and its orthologs in other plants. *HbSINA*4 showed a close relationship with the SINA family in millet, *B. distachyon*, and *O. sativa*, whereas the relationship was less close to the SINA family in *S. tuberosum* and *S. lycopersicum*. The complete SINA gene was cloned successfully in highland barley, named *HbSINA4*, and recorded given the accession No. KJ699391 in GenBank.

Expression patterns of the *HbSINA4* gene

The expression patterns of *HbSINA4* are presented in Figure 4. When the RSMC reached 33.4%, the expression of *HbSINA4* was up-regulated. With decreasing RSMC, gene expression also decreased. When the soil moisture content (C4) reached a normal level, expression was up-regulated. During drought stress, expression decreased when RSMC was drastically decreased. After 2 h water recovery, expression was again up-regulated over time. After 8 h, the expression got the pink when RSMC reached 33.4%. These results indicate that

the expression of *HbSINA4* was induced by drought stress and may be related to the drought resistance response in barley.

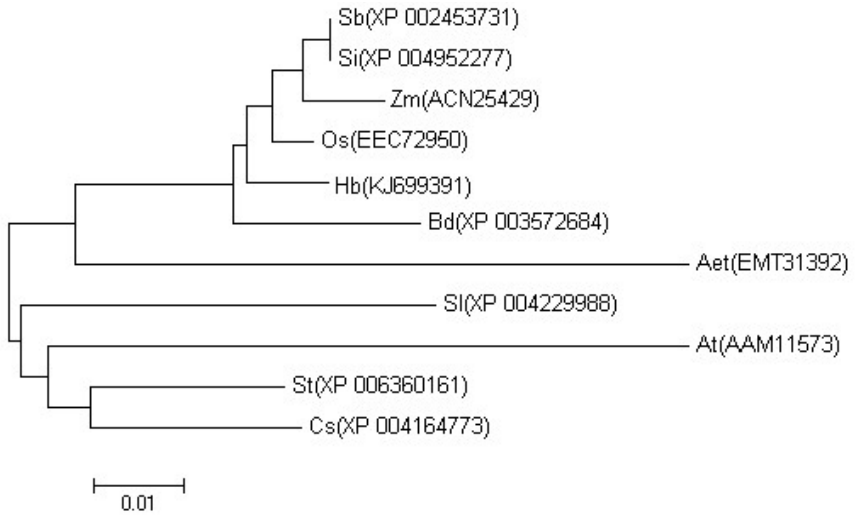

Figure 3. Phylogenetic tree of *HbSINA4* and other *SINA* from different genera. Sb = *Sorghum bicolor*; Si = *Setaria italica*; Os = *Oryza sativa*; Zm = *Zea mays*; Cs = *Cucumis sativus*; Bd = *Brachypodium distachyon*; Sl = *Solamum lycopersicum*; At = *Arabidopsis thaliana*; St = *Solanum tuberosum*; Hb = Tibetan hulless barley; Aet = *Aegilops tauschii*.

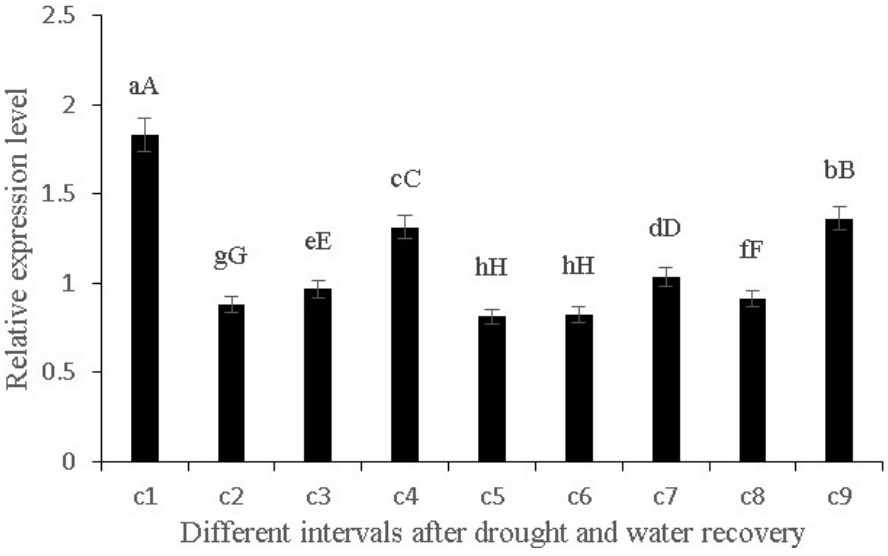

Figure 4. Real-time PCR analysis of the *HbSINA4* gene expression at different intervals during drought treatments. Drought stress at 6 levels, c1, 33.4%; c2, 27.5%; c3, 21.1; c4, 15.5%; c5, 9.8%; and c6, 4.8%, was examined. Watering recovery was tested at c7, 2 h; c8, 4 h; and c9, 8 h. One-way analysis of variance and the Duncan multiple range test were carried out, and the different lower case letter indicates significance at P = 0.05, while the different upper case letters indicate significance at P = 0.01.

DISCUSSION

SINA was first identified in *Drosophila melanogaster* (Carthew and Gubin, 1999) and represents an important gene family in plants. There are 5 members in *Populus alba*, 6 members in *O. sativa*, more than 6 members in *Zea mays*, and 2 members in Bryophytes (Wang, 2007). However, its function and biological process remain unclear. *SINA* has been shown to function as an *E3* (Xie et al., 2002). All *SINA* in plant contain a *SINA* domain and a *RING* domain, and the 2 domains are very conserved. Based on evolution analysis, plant SINA proteins are placed into 2 groups. Most group I proteins contain a motif 3 conserved domain at the C-terminus, while no group II proteins have this motif (Wang, 2007). Based on *Arabidopsis* transgenic plants, 2 members in group I were found to increase the length of the taproot, but reduce the distribution of the lateral root (Wang, 2007). In contrast, group II plants can reduce the length of the taproot. *SINAT5* in *Arabidopsis* has been thoroughly examined. As a single subunit, *SINAT5* can degrade NAC1, which is an important factor in lateral root formation. Over-expressed *SINAT5* reduces lateral root growth of *Arabidopsis*, whereas a mutant from conserve Cys in over-expressed *RING* domain will increase lateral root growth (Xie et al., 2002). Down-regulation of *SINAT5* allows the formation of lateral roots in *Arabidopsis*. Additionally, Fraire-Velázquez and Lozoya-Gloria (2003) obtained a cDNA that was very similar to *SINAT5* in bean. This gene may be induced by pathogenic bacteria and may play roles in the disease resistance response. Jaradat (1999) identified the *GhSINAH-1* gene, which is related to cotton fiber cell elongation. Colligating with the achievements of this gene, *SINA* in plants differ in functions, and may be involved in different physiological processes. However, these functions are not well-understood, with exception of *SINAT5* in *Arabidopsis*.

This study was based on previously unpublished results from transcriptome sequencing of barley under different drought stress conditions. In this study, we cloned *HbSINA4*, which was found to belong to group I because its motif 3 is conserved at the C-terminus. RT-PCR analysis indicated that *HbSINA4* expression changed in response to drought stress, and thus may play a very important role in drought resistance. Furthermore, this result suggested that *SINA* has diverse functions in plant. This is the first report to demonstrate that *SINA* is involved in drought stress. It remains unclear whether the response to drought is related to the regulation of the taproot and lateral root in *Arabidopsis*. In addition, it is unknown whether *HbSINA4* is involved in other physical processes. Therefore, further studies are needed to clarify the role of *HbSINA4* in plants.

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