Genetics and Molecular Research

Cloning and expression pattern analysis of *BkGRAS2* from *Betula kirghisorum*

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ABSTRACT. GRAS proteins are plant-specific transcription factors that are involved in the regulation of root and shoot growth. Here, we cloned *BkGRAS2* from *Betula kirghisorum* (abbreviated to Bk) and analyzed the physicochemical properties and expression pattern of the encoded protein. *BkGRAS2* had an open reading frame of 1614 bp encoding 537 amino acid residues. The deduced BkGRAS2 protein was hydrophilic, and it contained highly conserved VHIID and SAW motifs. *BkGRAS1* and *BkGRAS2* showed considerable sequence similarities. An expression analysis indicated that *BkGRAS2* was expressed in root, stem, and leaf, with the highest level in the leaf. Expression of *BkGRAS2* was increased following stress treatment with 0.6% $NaHCO_3$. Transient expression analysis of *GFP-BkGRAS2* in onion epidermal cells revealed that the BkGRAS2 protein was localized in the cytoplasm, but could also be detected in the nucleus. Our study provides the basis for future research on the role of the GRAS gene family in *B. kirghisorum.*

Key words: *Betula kirghisorum*; GRAS transcription factor; Expression pattern; $NaHCO_3$ stress; Subcellular localization

INTRODUCTION

Soil salinization as a result of natural factors and human industrial and agricultural activities is an increasingly serious ecological problem worldwide. In particular, soil salinization restricts the development of agriculture and the forestry industry and requires ecosystem restoration. In China, it is estimated that about 3.69 x 10^7 hm^2 of arable land is affected by soil salinization, i.e., approximately one-quarter of the available land, and that this area is increasing annually (Foyer and Noctor, 2000; Tester and Davenport, 2003). In addition to soils affected by human activities, there are areas with naturally high levels of soil salinity. For example, the Songnen Plain of the northeast region of China is one of the three largest areas of soda saline soil in the world (Pan et al., 2006). The physicochemical property analysis of saline soil indicated that pH was between 9.6 and 10.2 in this area, showing a strong base reaction (Wang et al., 2001).

Over the last two decades, considerable research effort has been devoted to investigation of the GRAS gene family, which encode plant specific transcription factors. The proteins have a conserved C-terminal domain and a highly variable N-terminal domain. The family has been divided into eight subfamilies, namely, DELLA, HAM, LISCL, PAT1, LS, SCR, SHR, and SCL3 (Tian et al., 2004). These transcription factors play an important role in the growth and development of plants (Schumacher et al., 1999; Llave et al., 2002; Stuurman et al., 2002; Gao et al., 2004; Sánchez et al., 2007), GA signal transduction (Peng and Harberd, 1997; Silverstone et al., 1997; Lee et al., 2002; Wen and Chang, 2002), light signal transduction (Bolle et al., 2000; Torres-Galea et al., 2006, 2013), and other biotic and abiotic stress processes. For example, in *Arabidopsis*, SCARECROW-LIKE 3 (*SCL3*) acts as a positive regulator to integrate and maintain a functional GA pathway by attenuating DELLA repressors in the *Arabidopsis* root, enabling root cell elongation and shoot tissue maturation (Heo et al., 2011; Zhang et al., 2011).

Our laboratory is interested in elucidating the salt tolerance mechanism in *Betula kirghisorum*, with the ultimate aim of cultivating salt tolerant varieties. Such varieties could be applied to the improvement of the saline alkali soils of the Northeast region of China, and improve the habitat for rare plants and overcome the low species diversity in these saline and alkaline areas. Unfortunately, little attention has been paid to GRAS genes in *B. kirghisorum*, although we did successfully clone a GRAS gene, *BkGRAS1* (Yang et al., 2013). In the present study, we have extended our studies of this species and successfully cloned a second GRAS gene, *BkGRAS2.* We investigated salt resistance of different organizations of *Betula kirghisorum* and examined *BkGRAS2* expression under salt stress conditions. The study has potentially important practical significance for soil improvement and environmental greening of the saline and alkaline areas of the Northeast region of China.

MATERIAL AND METHODS

Plant materials

One-year-old *B. kirghisorum* plants were obtained from the breeding garden of the Northeast Forestry University. Similar-sized plants (20-30 cm tall) with 15-20 leaves were used for the expression analysis. The plants were subjected to stress for different lengths of time, namely, 0, 12, 24 or 48 h; the stress was applied by watering the plants with 0.6% $NaHCO_3$.

Reagents and vector

DNA digestive enzymes, reverse transcription reagents, Ex Taq, and OMEGA gel extraction kit were purchased from TaKaRa Biotechnology (Dalian) Co., Ltd. The plasmid extraction kit and DH5α competent cells were purchased from Tiangen Biotech Ltd. All reagents were of analytical grade. For cloning, we used the *pMD18-T* vector; for expression analysis, we used the *pBI121-GFP* vector

Isolation of *BkGRAS2*

Primers of *BkGRAS2* clone were designed according to the sequence from the *Betula kirghisorum* transcriptome sequencing. Total RNA was extracted by the CTAB method from leaves of unstressed *B. kirghisorum* plants and subjected to agarose gel electrophoresis. cDNA synthesis was performed using M-MLV reverse transcriptase and an oligo(dT) primer. The cDNA sequence was amplified by PCR using the primers shown in Table 1.

Table 1. Primers used to clone *BkGRAS2*.

Primer	Primer sequence (5'→3')
BkGRAS2-F	ATTCTCCCCTTTGTTTCTTGGCTAA
BkGRAS2-R	CAGAGCAACCATTTTCACCTCCAAG

Bioinformatic and structural domain analysis of *BkGRAS2*

The physicochemical properties of the protein encoded by *BkGRAS2* were predicted using the online Expasy Protparam (http://web.expasy.org/protparam/). The gene domains were predicted using CDD online software (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The structural motifs of *BkGRAS2* were determined using the published information on GRAS family domains in *Arabidopsis thaliana* (Pysh et al., 1999).

Comparison of *BkGRAS1* and *BkGRAS2* at the nucleotide and protein levels

The nucleotide blast software (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) in NCBI was used to analyze differences between *BkGRAS1* and *BkGRAS2* nucleotide sequences. The relationship between BkGRAS1 and BkGRAS2 proteins were analyzed using the ClustalW2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

PCR analyses

Total RNAs from roots, stems, and leaves of *B. kirghisorum* were obtained by the CTAB method from tissues sampled after different periods of stress. Each RNA sample was treated with DNaseI and used to produce cDNA. The cDNA was diluted 5-fold and used as the template for PCR amplification with the primers shown in Table 2. The PCR products were separated on a 1.0% agarose gel and stained with ethidium bromide. Quantitative real-time PCR (qRT-PCR) assay was

performed using the same RNA samples and primer sets. The *Betula* 18S transcript was used as a control, and the relative levels of *BkGRAS2* in each sample were normalized against 18S transcript levels. SYBR green was used to monitor the kinetics of PCR product formation in qRT-PCR; each PCR assay was carried out three times. The qRT-PCR reaction conditions are outlined in Table 3, and 40 PCR cycles were performed: 94°C for 5s, 60°C for 34s. The relative quantification value of BkGRAS2 was calculated by the $2^{-\Delta\Delta Ct}$ method and the results from one representative experiment are shown.

Table 2. Quantitative Real-time PCR Primers for *BkGRAS2*.

Primer	Primer sequence (5'→3')
18S-F	ATCTTGGGTTGGGCAGATCG
18S-R	CATTACTC CGATCCCGAAGG
BkGRAS2-F	AGGCACT GGCTCAAGGAA
BkGRAS2-R	TGTAA GCACCCAATCGTT

Table 3. Reaction mixture for quantitative real-time PCR of *BkGRAS2*.

Component	Volume
2X TransStart® Tip Green qPCR SuperMix	10 µL
Passive Reference Dye (50X)	0.4 µL
Forward Primer (10 µM)	0.4 µL
Reverse Primer (10 µM)	0.4 µL
cDNA (5X)	2.0 µL
ddH_2O	up to 20 µL

Subcellular localization analysis

For the analysis of the subcellular distribution of the BkGRAS2 protein, the coding region of BkGRAS2 (without the termination codon) was inserted upstream of the GFP element of the *pBI121-GFP* vector. The fusion construct (*35S-BkGRAS2-GFP*), under the control of the cauliflower mosaic virus (CaMV) 35S promoter, was introduced into onion epidermal cells by the particle bombardment method using the PDS-1000 System (BioRad) at 1350 psi helium pressure. A blank 35S-GFP vector was used as a control. Onion cells were cultured for 24 h in the dark in a Tissue Culture room (Beijing Kingpeng Internatioal Hi-Tech Corporation) and then analyzed using a fluorescence microscope.

RESULTS AND DISCUSSION

Isolation of *BkGRAS2*

The GRAS gene family is widely distributed in plants. Currently, 33 GRAS transcription factors have been identified in *Arabidopsis thaliana*, mainly from the SCL (Pysh et al., 1999), GAI (Peng et al., 1997), RGA (Silverstone et al., 1998), and SCR sub-families (Laurenzio et al., 1996). Sixty GRAS family members are known in rice, such as *MOC1* (Li et al., 2003), *SLR1* (Ikeda et al., 2001), and *CIGR1/2* (Day et al., 2004). GRAS genes have been also isolated from maize (Lim et al., 2000), *Pinus* (Solé et al., 2008), *Medicago truncatula* (Kim and Nam, 2013).

RNA electrophoresis detection is shown in Figure 1. cDNA synthesis was performed using M-MLV Reverse Transcriptase and an Oligo(dT) primer. We designed clone primers in the 5' and 3' untranslated regions of *BkGRAS2.* The cDNA sequence was amplified by PCR using the primers (Table1). Sequencing after genetic transformation, we obtained the band of 1675 bp (Figure 2), in which the CDS (Coding sequence) was 1614 bp. Sequencing results indicated that the gene did not contain any introns in its coding region (Figure 3).

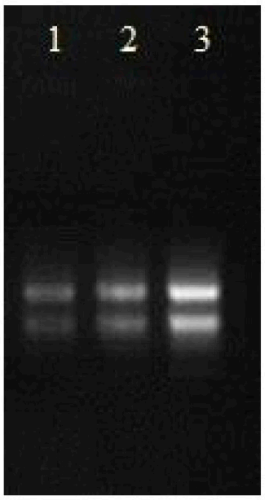


Figure 1. Electrophoretic detection of total RNA. After extraction from untreated (control) leaves (*lanes 1*, *2*, and *3*), RNA was detected on 1.0% agarose gels.

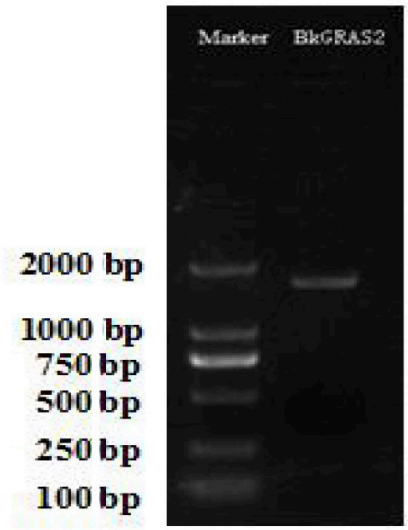


Figure 2. cDNA product of *BkGRAS2* produced by PCR amplification. The sequence was amplified using the primers *BkGRAS2*-F and *BkGRAS2*-R. Left lane: DM2000 size marker; right lane: *BkGRAS2* gene product.

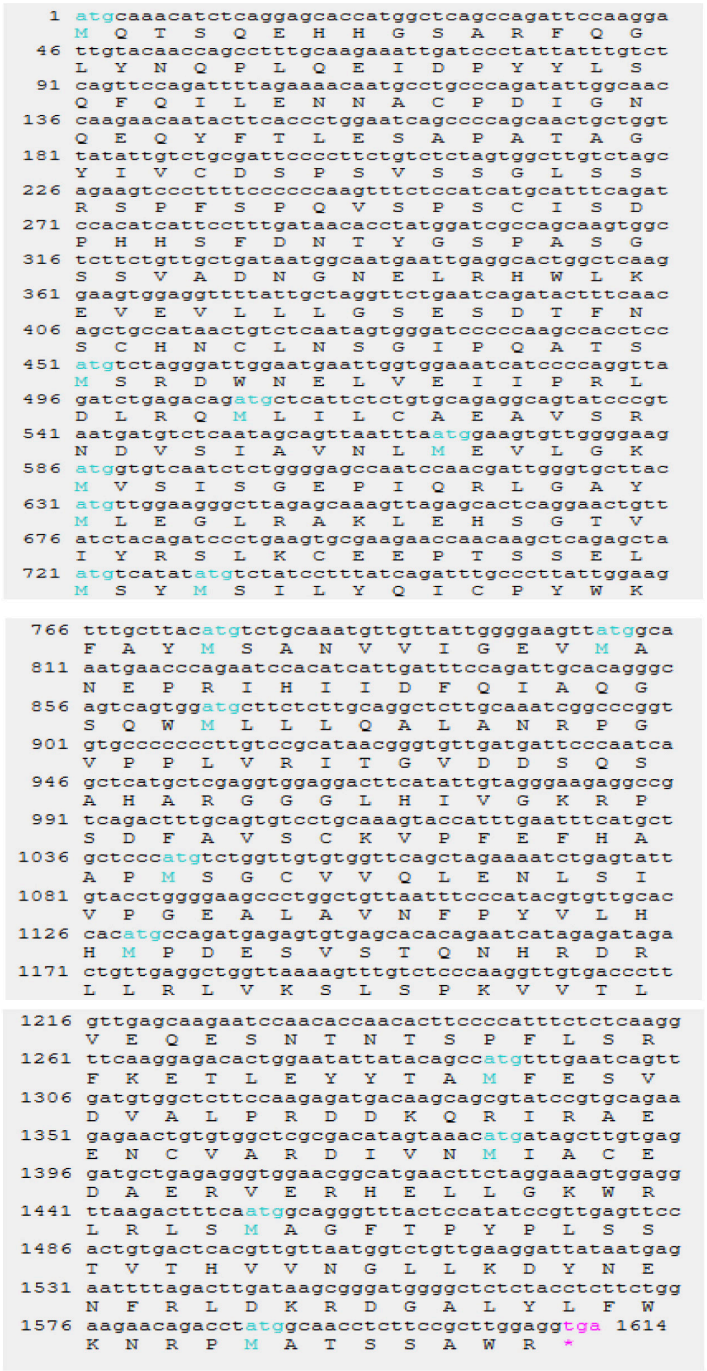


Figure 3. Analysis of the open reading frame of *BkGRAS2*. The open reading frame of *BkGRAS2* was determined using the ORF finder procedure (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) in NCBI. Codons marked in blue are initiation codon (first ‘‘atg’’) and nucleotides coding methionine (other ‘‘atg’’ codons). In pink, there is the termination codon.

Bioinformatics and structural domain analysis of *BkGRAS2*

Previously, according to the results of transcriptome sequencing our laboratory cloned the first GRAS gene from *Betula kirghisorum* and named it *BkGRAS1* (Yang et al., 2013). Nowadays, we cloned the second gene and use the NCBI CCD software to analyze the predicted protein of the isolated open reading frame which indicated that the gene belonged to the GRAS family (as shown in Figure 4). Therefore, we designated the gene as *BkGRAS2*. The open reading frame of *BkGRAS2* is shown in Figure 3. It is predicted to encode a protein of 537 amino acid residues with an estimated molecular mass of 60.0 kDa and a theoretical pI of 5.56. The grand average of hydropathicity was -0.223, indicating that the BkGRAS2 protein was hydrophilic.

Comparison of the deduced amino acid sequence of BkGRAS2 with *A. thaliana* GRAS proteins (Figure 5) indicates the presence of typical GRAS domains. For the VHIID motif, the P-N-H-D-Q-L residues were absolutely conserved, whereas the SAW motif was characterized by three pairs of absolutely conserved residues, R-E, W-G and W-W (Figure 5).

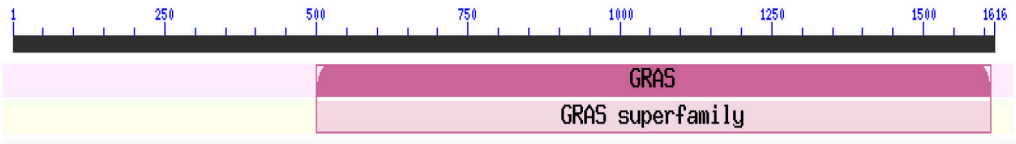


Figure 4. Schematic diagram of the *BkGRAS2* domain. Prediction of the gene domains using the CDD software (http://www.ncbi.nlm.nihgov/Structure/cdd/wrpsb.cgi).

Comparison of *BkGRAS1* and *BkGRAS2* nucleotide and protein sequences

A comparison of the open reading frames of *BkGRAS1* (Yang et al., 2013) and *BkGRAS2* showed they had equal lengths. Additionally, the two genes have many similarities in their physical and chemical properties. Therefore, we further characterized the relationship of the two genes at the nucleotide and protein levels using nucleotide blast and the ClustalW2 software (Figures 6 and 7). The nucleotide sequence comparison identified seven base pair changes in the two genes (Figure 6). The amino acid sequence comparison identified four different amino acids between BkGRAS1 and BkGRAS2 proteins (Figure 7). The differences between the two genes are summarized in Table 4.

Effect of 0.6% $NaHCO_3$ stress on *BkGRAS2* expression

In the shoots and roots of *Arabidopsis* seedlings subjected to osmotic and drought stress, *SCL13* expression was significantly increased (Guo et al., 2009). *HcSCL13* expression is induced in *Halostachys caspica* by salt stress, suggesting that expression of this gene is related to salt tolerance (Zhou et al., 2013). The HcSCL13 protein has a transcriptional activation domain indicating that it is a transcription factor (Zhou et al., 2014). Ma et al. (2010, 2011) cloned the *PeSCL7* gene from *Populus euphratica* and showed that its expression was induced by salt, drought stress treatment, and gibberellin (GA). Under salt stress conditions (350 mM NaCl), expression peaked after 3 h (Ma et al., 2011).

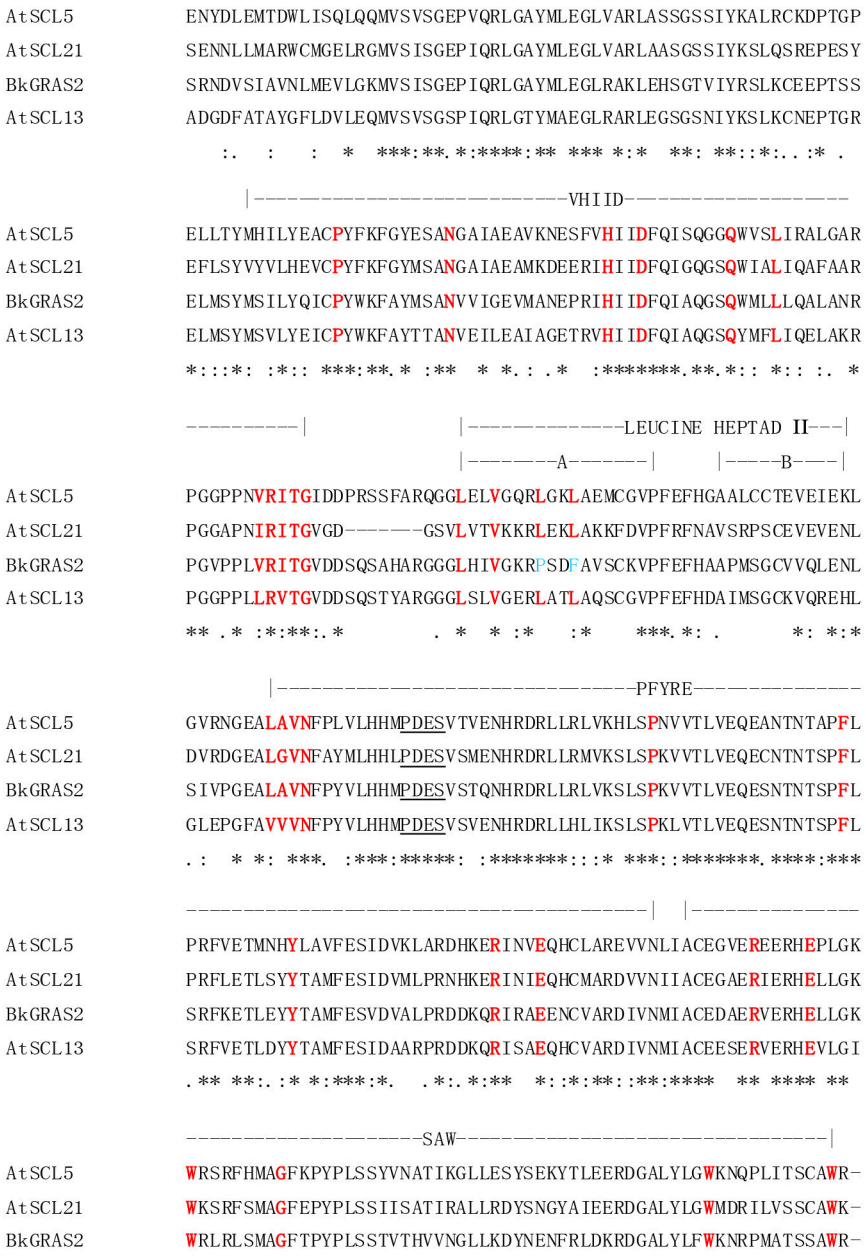


Figure 5. Alignment of the protein products of *Arabidopsis* GRAS genes and *Betula kirghisorum BkGRAS2*. The sequence alignment of the deduced amino acids identify highly conserved regions in BkGRAS2. The absolutely conserved residues within the VHIID, PFYRE, and SAW motifs are highlighted in bold red. Blue: in the A part of LEUCINE HEPTAD II motif, there were different amino acids between *Betula kirghisorum* and *Arabidopsis*. Underlined regions: in the literature (Pysh et al., 1999), some genes in *Arapbidopsis* did not contain PDES amino acids in this position. The BKGRAS2 gene was the same as AtSCL5, AtSCL13, and AtSCK21 genes, which contained PDES amino acids in this position.

Figure 6. Comparison of the nucleotide sequences of *BkGRAS1* and *BkGRAS2* indicates seven base pair changes.

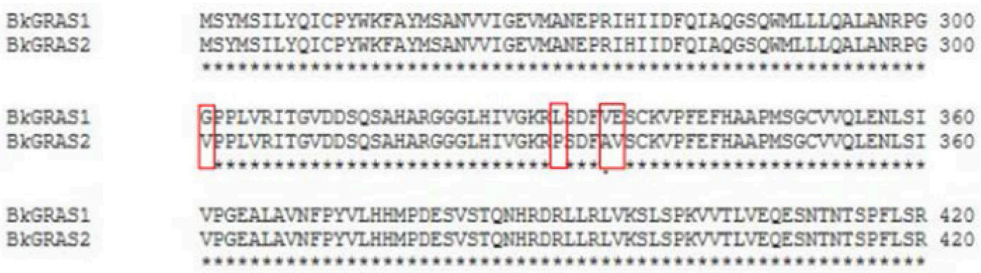


Figure 7. Comparison of amino acid sequences of BkGRAS1 and BkGRAS2 proteins. Four amino acids differences are present between the two proteins.

Table 4. Comparison of nucleotide and amino acid sequence differences between *BkGRAS1* and *BkGRAS2*.

Sequence number	Base change	Amino acid number	Amino acid change
454-456	TCG-TCT		
901-903	GGG-GTG	301	G-V
988-990	CTG-CCG	330	L-P
994-996	GAT-GAC		
1000-1002	GTG-GCA	334	V-A
1003-1005	GAG-GTG	335	E-V

In the present study, a qRT-PCR assay showed that *BkGRAS2* was expressed in all organs tested (roots, stems, and leaves) (Figure 8). Following exposure to stress (0.6% $NaHCO_3$) for different periods of time (0, 12, 24, and 48 h) expression levels varied. In the untreated control, *BkGRAS2* expression was highest in the leaf and lowest in the root (Figure 8A). Under stress conditions, *BkGRAS2* expression rose rapidly in the root, with a peak at 12 h (Figure 8B). By contrast, expression levels in the stem did not immediately rise, but reached a maximum at 48 h (Figure 8C). Compared to the control, *BkGRAS2* expression was greater in the leaves of treated plants at 12 and 48 h, although it was low at 24 h (Figure 8D). The increased expression of *BkGRAS2* after 0.6% $NaHCO_3$ stress treatment suggested that the gene is involved in salt stress responses.

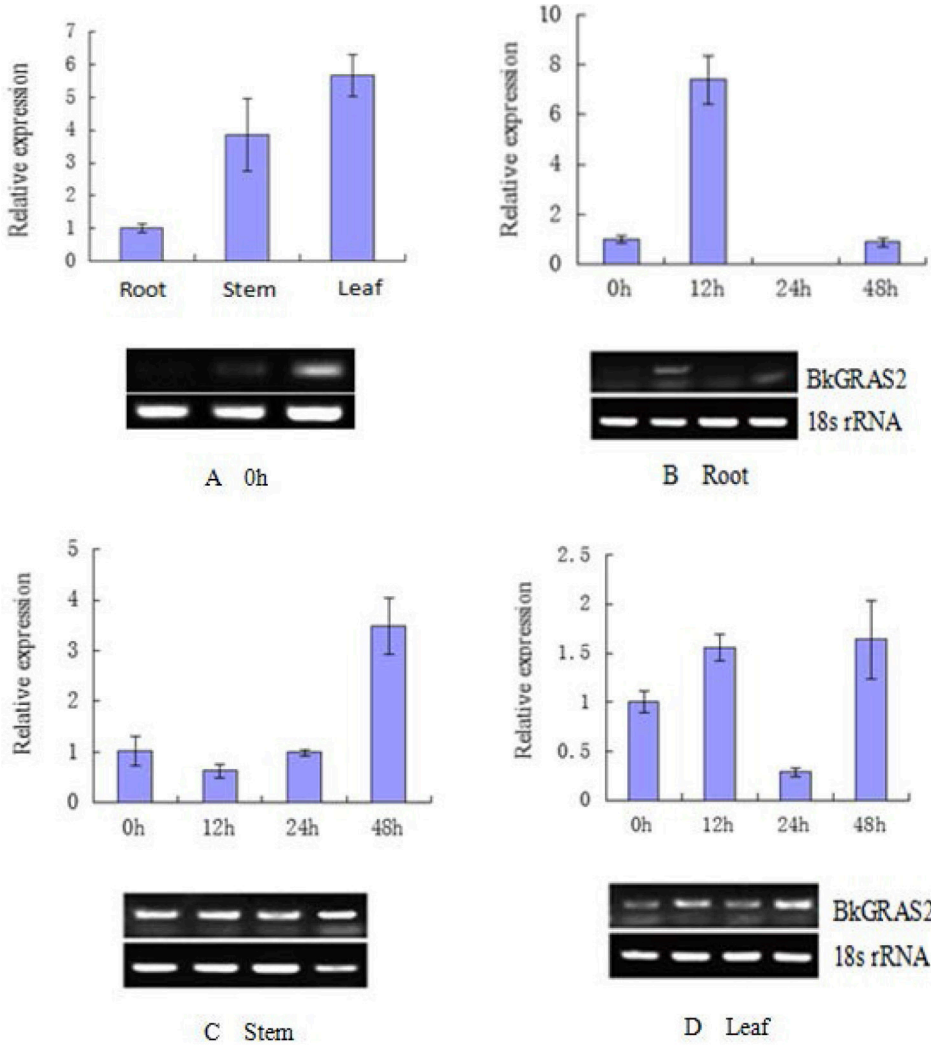


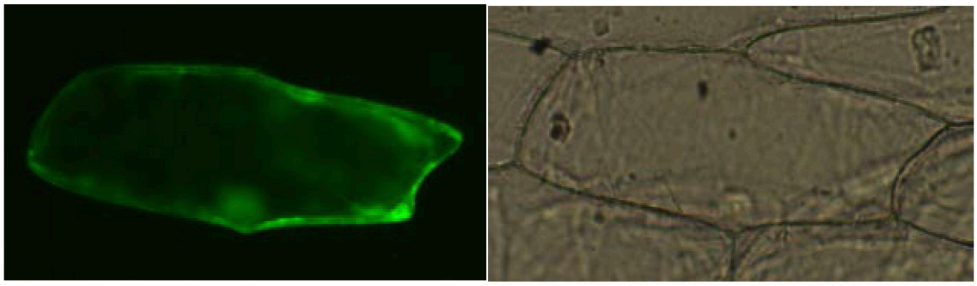
Figure 8. qRT-PCR and RT-PCR analyses of *BkGRAS2* expression. (**A**) Level of *BkGRAS2* transcripts under normal environmental conditions. The level of transcript in the root was set as 1.0. (**B**, **C**, **D**). Relative levels of expression in roots, stems, and leaves under 0.6% $NaHCO_3$ stress. Expression levels were normalized against 18S rRNA, and the level of transcript in the controls (0 h, without stress) was set as 1.0.

Intracellular distribution of BkGRAS2

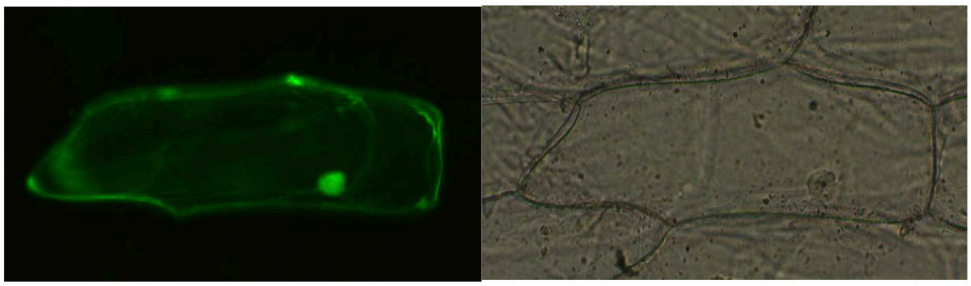
The intracellular distributions of a few GRAS proteins have been reported. NtGRAS1 (Czikkel and Maxwell, 2007), RGA (Silverstone et al., 1998), and RGL (Wen and Chang, 2002) have been shown to localize to the nucleus, while PAT1 is mainly present in the cytosol (Bolle et al., 2000).

To study the intracellular distribution of BkGRAS2, the coding region of *BkGRAS2* was fused to *GFP* under the control of a 35S promoter. The fusion plasmid (*35S-BkGRAS2-GFP*)

and the control vector (*35S-GFP*) were introduced into onion cells by particle bombardment, with untransformed onion cells serving as a control. GFP expression was examined by fluorescence microscopy after 24 h of incubation. We observed signals throughout cells transformed with the control plasmid (*35S-GFP*, Figure 9A). In cells transformed with the *35S-BkGRAS2-GFP* plasmid (Figure 9B), GFP could be detected in the nucleus and cytoplasm. Therefore, it is possible that BkGRAS2 plays an important role throughout the whole cell, in a similar manner as the SCL protein (Torres-Galea et al., 2006).



A. 35S-GFP



B. *35S-BkGRAS2-GFP*

Figure 9. Subcellular distribution of BkGRAS2 in onion epidermal cells. *p35S-GFP* (control) and *p35S-BkGRAS2-GFP* were transiently expressed in onion epidermal cells. Images were captured by fluorescence and light-field microscopy for the *p35-GFP* (control) plasmid (**A**) and the *p35S-BkGRAS2-GFP* plasmid (**B**).

CONCLUSIONS

Previous research showed that some GRAS genes respond to salt, drought, and low temperature stresses. Here, we used transcriptome sequences in databases to screen for a GRAS gene in the transcriptome of *B. kirghisorum*, and then cloned the gene and performed a bioinformatic analysis. We also examined the expression behavior of this gene under salt stress. The *BkGRAS2* gene identified here showed close similarity to *BkGRAS1* at the nucleotide and protein levels. Analysis of expression of *BkGRAS2* showed that it was induced by a 0.6% $NaHCO_3$ stress, and that the relative levels of expression varied with time and among tissues. Further experiments will be necessary to elucidate the specific mechanisms of this expression pattern.

The BkGRAS2 protein did not show localization, but it was distributed throughout the cell. This suggests that *BkGRAS2*, possibly in combination with other genes, plays a role in the nucleus,

and that after transfer, it also functions in the cytoplasm. The experiment provides fundamental data for the identification and analysis of members of the GRAS gene family in *B. kirghisorum*, and provides experimental information of value for developing more salt-tolerant varieties.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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