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Research article

GmSK1, an SKP1 homologue in soybean, is involved in the tolerance to salt and drought

Yanping Chen[a,b], Yingjun Chi[a,c], Qingchang Meng[b], Xiaolin Wang[a], Deyue Yu[a,*]

[a] College of Life Sciences/National Center for Soybean Improvement/Nanjing Agricultural University, Nanjing, 210095, China
[b] Institute of Food Crops, Jiangsu Academy of Agricultural Sciences, Nanjing, 210014, China
[c] College of Agro-grassland Science, Nanjing Agricultural University, Nanjing, 210095, China

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ABSTRACT

In plants, various proteins are regulated by the ubiquitin-mediated system in response to different environmental stresses, such as drought, cold and heat. The Skp1-Cullin-F-box (SCF) complex, one of the multisubunit E3 ligases, has been shown to be involved in abiotic response pathways. In this study, *Glycine max SKP1-like 1* (*GmSK1*), which had the typical characteristics of an SKP1 protein, with an alpha/beta structure, targeted to the cytoplasm and nucleus, was isolated from soybean [*Glycine max* (L.)]. *GmSK1* was constitutively expressed in all the tested tissues, especially in the roots. Furthermore, the expression of *GmSK1* was simultaneously induced by abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA), NaCl, low temperatures and drought, which suggests important roles for *GmSK1* in plant responses to hormone treatments and abiotic stress. *GmSK1*-overexpressing transgenic tobacco (*Nicotiana tobacum* cv. Samsun) plants showed enhanced tolerance to high salinity and drought stress; exhibited significantly reduced inhibition of growth, greenness and water loss; and exhibited increased MDA accumulation compared with wild-type controls. Our results suggest that *GmSK1* might play a role in the crosstalk between ubiquitination and abiotic stress responses in plants.

1. Introduction

Ubiquitination plays an important role in various cellular responses, such as regulation of the cell division cycle, hormonal signaling and stress responses, in plants. The ubiquitination process requires a three-step enzymatic cascade: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3) (Seo et al., 2012). E3 ligases can be largely divided into two groups based on structure: single-subunit E3 ligases and multisubunit E3 ligases (Hotton and Callis, 2008).

In previous studies, multisubunit E3 ligases have been shown to be involved in abiotic stress response. To avoid growth defects triggered by abiotic stress, plants have developed various mechanisms to sense water limitation and salt stress. Stress signaling is largely composed of abscisic acid (ABA)-independent and ABA-dependent pathways (Nguyen et al., 2016). A number of studies have examined the relationship between Skp1-Cullin-F-box (SCF) and ABA signaling. DWD hypersensitive to ABA (DWA) proteins, DWA1 and DWA2, function as substrate receptors for SCF and play a negative role in ABA responses. Both of these proteins exhibited hypersensitive phenotypes in response to ABA and salt stress (Lee et al., 2010). DWA1 and DWA2 were able to associate with ABA Insensitive 5 (ABI5) in vivo, and were directly responsible for ABI5 degradation mediated by the 26S proteasome-dependent pathway. This finding indicates that ABI5 is a direct target of E3 ligase complexes, which utilize DWA1 and DWA2 as substrate receptors (Lee et al., 2010). E3 ligases can be responsive to abiotic stress mediated by the ABA-dependent pathway (Lee and Kim, 2011).

One of the best characterized and most important multisubunit E3 ligases is the SCF protein complex. The S-phase kinase-associated protein 1 (SKP1) is a major component of the SCF complex that facilitates ubiquitin-mediated protein degradation in plants (Hotton and Callis, 2008). SKP1 serves as an adaptor that links the Cullin1 and F-box proteins (Li et al., 2012; Hong et al., 2013). In *Arabidopsis thaliana* (Arabidopsis), there are 21 predicted SKP1-like genes (ASK) (Kong et al., 2007). The ASK1 gene is essential for male meiosis and ASK2 has similar functions to ASK1. Both ASK1 and ASK2 regulate embryo development (Yang et al., 1999; Liu et al., 2004). SKP1 plays an important role in regulating hormone signaling pathways, such as the auxin (Li et al., 2006), jasmonic acid (JA) (Xie et al., 1998) and gibberellic acid (McGinnis et al., 2003) signal transduction pathways. Some reports have shown that SKPs can regulate stress tolerance and participate in the ABA signaling pathway. Overexpression of *PSKP1*, an *SKP* from

* Corresponding author.
E-mail address: dyyu@njau.edu.cn (D. Yu).

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Paeonia suffruticosa, confers salinity tolerance to Arabidopsis (Li et al., 2006). Expression of SSKs (SSK4, SSK7 and SSK12) was found to be responsive to different kinds of abiotic stress, including heat and salinity, with expression levels of nearly 50% of analyzed SSKs being up-regulated by ABA and salicylic acid (SA) treatments in wild tomato plants (Zhang et al., 2015). It has been reported that the *GsSKP21* gene participates in plant alkalinity tolerance regulation and in the ABA signal pathway. *GsSKP21* overexpression resulted in ABA insensitivity during seed germination and early seedling growth stages (Liu et al., 2015).

Soybean [*Glycine max* (L.)] is a major source of vegetable protein and edible oil. However, the production of soybean is under threat by many abiotic stress factors, such as drought, salinity and osmotic stress (Manavalan et al., 2009). In this study, *Glycine max SKP1*-like1 (*GmSK1*), encoding an S-phase kinase-associated protein 1, was isolated from soybean. The expression of *GmSK1* was induced by various stress factors and hormones, such as salt, drought, low temperatures, ABA, JA, and SA. Upon overexpressing *GmSK1* in tobacco, the transgenic plants showed salt and drought tolerance, which suggested that *GmSK1* regulates abiotic tolerance via ubiquitin-mediated pathways in plants.

2. Material and methods

2.1. Plant materials and stress treatments

The soybean cultivar 'Nannong 99-10', which was bred by the National Center of Soybean Improvement, Nanjing Agricultural University, was used in this study. Roots, stems, leaves at the three-trifoliolate stage, flowers, immature pods, and seeds at 25 days after flowering were collected. Hormone and abiotic treatments were performed at the five true-leaf stage. For ABA, JA, and SA treatments, soybean seedlings were placed in solutions of 100 μM ABA, 100 μM JA, of 100 μM SA. For salt stress, the seedlings were placed in solutions of 150 mM NaCl. For drought treatment, the seedlings were placed in solutions of 20% PEG6000. For low-temperature treatment, seedlings were placed in a 4 °C growth chamber. To test the expression pattern of *GmSK1* during the early phase of the stress response (0–48 h) after treatment for different time periods, namely, 0 h, 2 h, 6 h, 12 h, 24 h and 48 h, leaves were selected for sampling. The samples were then frozen in liquid nitrogen and stored at −80 °C for further analysis.

2.2. Isolation of the GmSK1 gene

Total RNA was extracted from soybean leaves using the RNA Plant Extraction Kit (TIANGEN DP419). The sequences of the 5′ and 3′ terminal ends of *GmSK1* were obtained by rapid amplification of cDNA ends (RACE) using the BD SMARTer RACE cDNA Amplification Kit (Clontech). By sequence assembly of the EST, 5′ terminal and 3'terminal RACE sequences, the full-length cDNA of GmSK1 was amplified by PCR with the primers GmSK1-F and GmSK1-R. The primers used in this study are listed in Table S1.

2.3. Bioinformatics analysis

Nucleotide sequence analysis was performed using the NCBI ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and BioXM 2.6 software. The amino acid sequence was analyzed by using ProtParam (http://web.expasy.org/protparam/). By using SMART (http://smart.embl-heidelberg.de/) and the Pfam database (http://Pfam.wustl.edu), the functional protein domains were predicted. Homologous sequences of GmSK1 were identified using the Blastp search program of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). Molecular phylogenetic analysis was constructed by the maximum likelihood method using MEGA6. Multiple sequence alignments were conducted with BioEdit software.

2.4. Gene expression

Total RNA was extracted from various tissues using the RNA Plant Extraction Kit (TIANGEN DP419). First-strand cDNA was generated from total RNA using the PrimeScript™ Π 1st Strand cDNA Synthesis Kit (TaKaRa) in accordance with the manufacturer's instructions. Real-time quantitative PCR analysis was performed to determine the expression profiles of *GmSK1* by using the primers GmSK1-qPCRF and GmSK1-qPCRR (Table S1). The soybean *Tubulin* gene (GenBank AY907703) was used as a reference in the experiment (primers TubF and TubR, Table S1).

2.5. Subcellular localization of GmSK1

The full-length *GmSK1* coding region was fused to the pBI121-GFP vector under the control of the CaMV35S promoter. The primers used for amplification of the full-length region were GmSK1-BF and GmSK1-BR (Table S1), in which the termination codon of *GmSK1* was removed. Transient expression of the pBI121-GmSK1-GFP fusion construct and the pBI121-GFP control vector were each introduced into onion epidermal cells by *Agrobacterium*-mediated transformation. Transformed cells were cultured on MS medium for 16–24 h and observed under a confocal microscope (Leica Microsystem, Heidelberg, Germany).

2.6. Tobacco transformation

GmSK1 full-length cDNA was inserted into the binary vector pBI121 in the sense orientation. The construct was introduced into tobacco (*Nicotianatobacum* cv. Samsun) using *Agrobacterium*-mediated transformation. Transgenic plants were selected on MS medium containing 100 μg ml^{-1} kanamycin.

2.7. Salt- and drought-stress tolerance analysis of transgenic plants

Seeds of both wild-type and *GmSK1* transgenic tobacco were surface sterilized, sown on 1/2 MS medium containing 150 mM NaCl. The length and number of roots were measured after 30 days. For fresh weight measurement, seeds were grown in normal soil for 2 weeks. Then, the plants were transferred to soil containing 20% NaCl, and the fresh weights were measured after 30 days. To test the drought response, plants were initially grown in soil with normal watering for 2 weeks. Seedlings of both wild-type and *GmSK1* transgenic tobacco at the two-leaf period were transplanted, and given no water for 30 days, and then, the chlorophyll content, water-loss ratio and malondialdehyde (MDA) content were measured.

3. Results

3.1. Isolation and sequence analysis of GmSK1

An alfalfa (*Medicago sativa*) SKP1-homolog protein, which was highly differentially expressed during seed development in soybean, was identified by MALDI-TOF-MS by querying the peptide mass finger printing data in the NCBI database by using Profound (http://www.prowl.rockefeller.edu) (Zheng et al., 2008). Using the alfalfa SKP1-homolog protein sequence as a probe, we isolated the soybean expressed sequence tag (EST) assembly sequence (TC204343) from the soybean EST database. Based on TC sequence information, 271 bp and 336 bp sequences of the 5′ and 3'ends, respectively, were then obtained via RACE (Fig. S1A). By sequence assembly and PCR verification, a 1125-bp full-length cDNA sequence of the alfalfa SKP1-homolog was cloned and named *GmSK1* (Fig. S1A). Sequence analysis revealed that this gene contained a 468-bp open reading frame, encoding a polypeptide of 155 amino acids with a predicted molecular mass of 17.49 kDa (pI 4.55). The predicted GmSK1 protein contained a PEST motif, which is rich in proline (P), glutamic acid (E), serine (S) and