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Research article

GsSNAP33, a novel *Glycine soja* SNAP25-type protein gene: Improvement of plant salt and drought tolerances in transgenic *Arabidopsis thaliana*

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ABSTRACT

The N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) superfamily, specifically the SNAP25-type proteins and t-SNAREs, have been proposed to regulate cellular processes and plant resistance mechanisms. However, little is known about the role of SNAP25-type proteins in combating abiotic stresses, specifically in wild soybean. In the current study, the isolation and functional characterization of the putative synaptosomal-associated SNAP25-type protein gene *GsSNAP33* from wild soybean (*Glycine soja*) were performed. *GsSNAP33* has a molecular weight of 33,311 Da and comprises 300 amino acid residues along with Qb-Qc SNARE domains. Multiple sequence alignment revealed the highest similarity of the GsSNAP33 protein to GmSNAP33 (91%), VrSNAP33 (89%), PvSNAP33 (86%) and AtSNAP33 (63%). Phylogenetic studies revealed the abundance of SNAP33 proteins mostly in dicotyledons. Quantitative real-time PCR assays confirmed that *GsSNAP33* expression can be induced by salt, alkali, ABA and PEG treatments and that *GsSNAP33* is highly expressed in the pods, seeds and roots of *Glycine soja*. Furthermore, the overexpression of the *GsSNAP33* gene in WT *Arabidopsis thaliana* resulted in increased germination rates, greater root lengths, improved photosynthesis, lower electrolyte leakage, higher biomass production and up-regulated expression levels of various stress-responsive marker genes, including *KINI*, *COR15A*, *P5Cs*, *RAB18*, *RD29A* and *COR47* in transgenic lines compared with those in WT lines. Subcellular localization studies revealed that the GsSNAP33-eGFP fusion protein was localized to the plasma membrane, while eGFP was distributed throughout whole cytoplasm of onion epidermal cells. Collectively, our findings suggest that *GsSNAP33*, a novel plasma membrane protein gene of *Glycine soja*, might be involved in improving plant responses to salt and drought stresses in *Arabidopsis*.

1. Introduction

Vesicle trafficking is a fundamental phenomenon for growth and development, cell homeostasis and membrane fusion events in plants. In eukaryotes, these processes are carried out by a superfamily of protein receptors known as (SNAREs) (Sutter et al., 2006). Previous reports have highlighted the conventional housekeeping activities of this protein family associated with vesicle trafficking (Hong, 2005). However, recent studies involving genetic screening,

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phylogenetic analyses and yeast complementation tests have discovered several SNARE protein family members that are involved in multiple biological tasks (Surpin and Raikhel, 2004). A summarized list of these functions, which include environmental responses, stomatal movements, shoot gravitropism, sensitivity to salt and drought, signal transduction responses, control of non-SNARE proteins (specifically ion channels), pathogen defense, symbiosis and maintenance as well as some additional roles in membrane trafficking have been reported (Lipka et al., 2007). Functional classification has categorized SNAREs as target membrane-associated SNAREs (t-SNAREs) and vesicle-associated SNAREs (v-SNAREs) (Söllner et al., 1993); however, structural classification has divided SNARE proteins into Q and R groups, depending on the glutamine or arginine residues in the center of the SNARE domain (Fasshauer et al., 1998, 2003). Typically, t-SNAREs correlate with Q-SNAREs and v-SNAREs with R-SNAREs; Q-SNAREs can be further classified as Qa, Qb, and Qc SNAREs (Bock et al., 2001). Interestingly, SNAP25-like proteins occupy a special place due to the presence of a Qb-Qc SNARE motif in the individual polypeptide chain, which quite possibly emerged due to an ancient gene fusion process (Bennett et al., 1992).

The genomes of monocotyledons and dicotyledons encode an exceptionally large repertoire of SNARE proteins compared with those of other eukaryotes (Sutter et al., 2006). A number of t- and v-SNAREs that are homologous to those of yeast and animals have been found in *Arabidopsis* plants. A few of these SNAREs were discovered to be yeast and mammalian orthologs, while other SNAREs were detected only in *Arabidopsis*. The SNAP25-like proteins of plasma membrane origin are important members of SNAREs; these proteins were first reported in the mammalian neuron and are encoded by three genes, namely, *SNAP33*, *SNAP30* and *SNAP29*. SNAP proteins primarily play vital roles in cytokinesis. *Arabidopsis* plants also contain *SNAP33*, *SNAP30* and *SNAP29* genes that encode proteins of SNAP25-like group. Of these proteins, AtSNAP33 (a plasma membrane protein) was expressed ubiquitously in all tissues of *Arabidopsis* in high amounts; this protein is likely involved in various essential cellular processes, including vesicle secretion and cell division events. Due to these properties, AtSNAP33 has emerged as a candidate protein for working with SNARE complexes in driving immunity responses in *Arabidopsis* (Heese et al., 2001). The *AtSNAP33* gene is also involved in a number of abiotic and biotic stresses, as its expression level changes under both the inoculation with pathogens and hormonal treatments (Wick et al., 2003). In addition, wind and mechanical stresses play significant roles in increasing transcript levels of *AtSNAP33* in *Arabidopsis thaliana* (Wick et al., 2003). More recently, studies unveiled interactions between the AtSNAP33 protein and the tobacco homologs *NtSYP121* (a Qa-SNARE) and *NtSYR1*, which are involved in ABA, salt stress, wound stress responses and SNARE-mediated vesicle trafficking in tobacco leaves (Geelen et al., 2002; Kargul et al., 2001; Leyman et al., 2000). However, there is no direct evidence in the literature about involvement of soybean SNAREs in salinity-induced vesicle trafficking and osmotic responses. *Glycine soja* is a wild soybean species with strikingly high tolerance to salt. Seeds of this plant can usually grow and germinate in soils with 0.9% salt, as reported by Ji et al. (2006). Moreover, the growth rate of *Glycine max*, a commercially cultivated species, is severely hindered in soils with 0.3% salt content (Qiao et al., 2001). Hence, *G. soja* is considered an ideal crop for studying salt and drought stress signaling networks.

No study in the literature exists regarding the participation of the *SNAP33* gene of *G. soja* in abiotic stress responses. Therefore, we are presenting here for the first time the functional characterization of the SNAP25-type SNARE protein gene *GsSNAP33* using transgenic approaches and the evaluation of its ability to provide salt and drought tolerance using morphological, molecular, and biochemical assessments. These results could pave the way for understanding the potential applications of the *GsSNAP33* gene in engineering drought- and salt-tolerant soybean plants.

2. Materials and methods

2.1. Growth conditions

Wild soybean (*G. soja* (07256)) seeds were obtained from the Jilin Academy of Agricultural Sciences, Changchun, China. Soybean seeds were surface sterilized with 98% sulfuric acid for 10 min followed by 5–7 washings with distilled water, after which the seeds were placed in darkness (2–4 days) to break seed dormancy. Seedlings were then germinated in 1/4-strength Hoagland solution and sown at 24–26 °C and at 60% relative humidity under a 16-h light/8-h dark cycle provided by a SON-T ARGO 400-W light source with a constant illumination of 30,000 lx. WT *Arabidopsis* seeds of the Columbia ecotype were purchased from the Arabidopsis Stock Center (Nottingham, UK). *Arabidopsis* seeds were subsequently germinated and sown under ideal hydroponic conditions reported previously (Tocquin et al., 2003). To perform gene expression level analyses under various abiotic stress conditions (salt, alkali, osmotic and ABA), the roots of 3-week-old soybean seedlings were immersed in 1/4-strength Hoagland solution saturated with 200 mM NaCl, 50 mM $NaHCO_3$, 30% (w/v) PEG6000 or 100 μmol/L ABA. Root and leaf samples were collected after the following time intervals for each treatment: 0, 3, 6, and 12 h. For the determination of gene expression levels in *A. thaliana* under salt and osmotic stress, 2-week-old *Arabidopsis* seedlings were dipped in hydroponic nutrient solution saturated with 200 mM NaCl and 350 mM mannitol. Root samples were collected after following time intervals for each treatment: 0, 3, and 6 h. Samples were frozen immediately in liquid nitrogen and subsequently stored for RNA extraction at -80 °C.

2.2. GsSNAP33 gene isolation and bioinformatics analysis

Plant RNA was isolated from 3-week-old seedlings of *G. soja* by the use of a plant mini kit (Qiagen, Valencia, CA, USA). One microliter of RNA was used to generate cDNA with the use of a reverse-transcriptase kit (SuperScript III, Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. The *GsSNAP33* full-length CDS sequence was PCR amplified using the gene-specific forward (F 5′- CCTCCAACTCTGACCTTTCTTTACG -3′) and reverse primers (R 5′- GATGGACACCCAATTTGCTTGA -3′) to yield a 903-bp fragment containing the *GsSNAP33* cDNA. Error-free PCR amplicons were further recombined into a pGEM-T vector and transformed into a DH5α strain of *E. coli* (Promega, Madison, WI, USA) for the sequencing of desired gene fragments. Sequence similarity was investigated using the BLAST program with the GenBank database and default parameters available at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). In addition, queries to determine homologs were performed using the BLASTp program of Phytozome (http://www.phytozome.net/soybean), and multiple sequence alignment analysis was executed using Clustal X. The phylogenetic tree was constructed in accordance with neighbor-joining method and presented using MEGA 4.0 software using the amino acid sequences, as described by Kumar et al. (2008).

2.3. Quantitative RT-PCR assays

Total RNA isolation and synthesis of cDNA were performed as described previously in the materials and methods section. cDNA quality was first assessed using PCR. *ACTIN* (*Arabidopsis*) and *GAPDH*