Gene 536 (2014) 163–170

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Heterologous expression of an uncharacterized universal stress protein gene (*SbUSP*) from the extreme halophyte, *Salicornia brachiata*, which confers salt and osmotic tolerance to *E. coli*

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ARTICLE INFO

Article history:
Accepted 9 November 2013
Available online 27 November 2013

Keywords:
Abiotic stress
Halophytes
Osmotic tolerance
Salt stress
Salt tolerance

ABSTRACT

Salicornia brachiata is an extreme halophyte considered to be a rich source of stress responsive genes and an EST database revealed that 30% of its genes are uncharacterized. In order to ascertain its function, a gene (Sal-E-56) of unknown function was made full length using RACE, cloned and characterized. The full length gene (873 bp; accession no. KF164282) contained an open reading frame (ORF) of 486 bp encoding for a protein that belongs to the universal stress protein (USP) family that was named *SbUSP*. The *Sb*USP interacted with adenosine monophosphate and exhibited characteristic motifs, phosphorylation, glycosylation and ATP binding sites. Further, *in-silico* analyses suggested a probable role in metabolic process of phosphate-containing compounds including signal transduction. *In planta* transcript profiling exhibited a significant expression response (7.8-fold) to salt stress, additionally abundant of *SbUSP* transcripts were observed during drought, heat and cold stress, reaching a maximum increase of 3.66-, 2.64- and 2.14-fold, respectively, at 12 or 24 h. The heterologous expression of this gene in *Escherichia coli* provided enhanced stress tolerance and recombinant cells have higher growth rate compared to vector alone and showed growth at up to a 10^{-5} dilution in the spot assay. It was predicted that *SbUSP* may be directly involved in tolerance mechanisms or function as a molecular switch (signaling molecule) to activate the stress adaptive mechanisms. However, further investigation will be required to determine its role as a molecular switch and mode of action during stress.

1. Introduction

Abiotic stresses are major threats to agriculture and the environment. Salinity and drought are the abiotic environmental factors which adversely affect growth, limit productivity and geographic distribution of plants (Pardo, 2010). Salinity has an immediate effect on metabolism, creating hyperosmotic conditions and causing ion imbalances leading to oxidative damage in plant cells (Munns and Tester, 2008). Salinization is a global problem and about 800 million ha of land world-wide are affected (FAO, 2008), primarily due to scanty rain fall, uncontrolled usage of ground water and irregular irrigation.

Due to their ability to tolerate and live in the presence of high salt concentrations, halophytes have attracted the attention of researchers in an effort to understand the molecular mechanisms of salt tolerance (Flowers and Colmer, 2008). Halophytes constitute about 1% of the total world flora but are subjected to diverse stresses. Halophytes have evolved advanced mechanisms such as selective ion uptake or exclusion, compartmentalization, synthesis of osmoprotectants or antioxidants, activation or diversion of photosynthetic and/or energy metabolism, cellular modification, hormonal changes and expression of gene or gene cascade to cope with salinity-induced stresses (Riadh et al., 2010).

Genetic engineering is the preferred technique for creating enhanced salt tolerant plants. Although there are number of genes known to induce salinity tolerance upon introduction into plants, there is an ongoing search for novel genes from any genetic sources. Stress responsive genes of unknown function have been identified in different plants such as the *Hsdr4* gene from wild barley (Suprunova et al., 2007), the *AlSAP* gene from the halophyte grass *Aeluropus littoralis* (Saad et al., 2010), *MusaDHN-1* gene from banana cv. *Karibale Monthan* (Shekhawat et al., 2011), uncharacterized JAZ family genes belonging to *Glycine soja* (Zhu et al., 2012), an uncharacterized stress-induced sugarcane gene (Begcy et al., 2012) and the CBL-interacting protein kinase (*GhCIPK6*) gene from cotton (He et al., 2013).

Halophytes are considered as a rich source of salt responsive genes that play an important role in engineering stress tolerance into glycophytes (Rajalakshmi and Parida, 2012). Among halophytes, *Salicornia brachiata* is an extreme halophyte that inhabits salt marshes and requires NaCl for growth (even for tissue culture; Joshi et al., 2012). Adaptation to extreme salt conditions makes *Salicornia* a potential

Abbreviations: EST, expressed sequence tag; GST, glutathione S-transferase; ICP, inductive coupled plasma; qRT PCR, quantitative real time PCR; RACE, rapid amplification of cDNA ends; USP, universal stress protein.

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http://dx.doi.org/10.1016/j.gene.2013.11.020

candidate for stress responsive genes (Chaturvedi et al., 2012; Jha et al., 2011, 2013; Singh et al., 2013). Additionally, this plant has nutritional value, contains unique oligosaccharides and is eaten as salad greens (Jha et al., 2012; Mishra et al., 2013; Tiwari et al., 2013).

An expressed sequence tag (EST) database from *S. brachiata* subjected to salt stress was developed that contains ~30% genes which yet are to be characterized from which a gene encoding a protein belonging to the universal stress protein (USP) family was cloned and characterized. Stress associated protein (SAP) encoding genes are considered novel regulators of stress responses in plants (Giri et al., 2013). Expression was analyzed *in planta* under varying abiotic stresses as well as during heterologous *in-vivo* expression in *E. coli* under different stresses to determine its function. These uncharacterized genes may play a key role in salt tolerance mechanisms and could be utilized for the genetic engineering of crops. Uncharacterized genes are valuable resources and their functional validation will provide new insight into the molecular mechanisms of salt tolerance.

2. Materials and methods

2.1. Isolation and cloning of SbUSP gene

A *Salicornia* salt stress inducible EST database contained approximately 30% uncharacterized gene sequences from which the Sal-E-56 gene clone (EB484838) of unknown function was selected for the study. Seeds of *S. brachiata* Roxburgh were germinated in plastic pots containing garden soil under natural conditions. One month old seedlings were harvested and total RNA extracted using the GITC method (Chomczynski and Sacchi, 1987). Poly A^+ mRNA was isolated by magnetic separation using biotinylated oligo-dT primers, immobilized with streptavidin linked paramagnetic beads (Promega, USA). The partial EST sequence of clone (EB484838) was used to design gene specific primers for RACE (Rapid amplification of cDNA ends).

The first strand of cDNA was prepared using polyA^+ RNA (5 μg), GSP1 primer (100 nM; 5′-GAC AGA CCG TAA ACA AAG CC-3′), dNTPs (400 μM) and MLV reverse transcriptase (200 units; Super script II RT) at 42 °C and unincorporated ingredients were removed using SNAP columns (Invitrogen, USA). Homopolymeric tails (TdT tailing) were added to the 3′end of cDNAs by incubating at 37 °C for 10 min with TdT (Terminal deoxynucleotidyl transferase) and dCTP (200 μM) to create binding sites for the abridged anchor primer. The tailed cDNA (GSP1 & 2) was amplified by PCR using nested (GSP2 and GSP3; 400 nM) primers (GSP2: 5′-AGA ACC GCC CTT TTG ATA G-3′, anneals 3′ to GSP1; GSP3: 5′-GCA TCA CCT TCT ACT ATT TCT G-3′ anneals 3′ to GSP2), respectively, dNTPs (200 μM), *Taq* DNA polymerase (20 units) and a homopolymer containing an abridged anchor primer (400 nM) which binds to the homopolymeric tail. The cDNA for 3′-RACE was synthesized by reverse transcription carried out at 42 °C for 50 min, using Superscript II RT with 3′-RACE R1 primer which contained an oligo $(dT)_{17}$-adaptor site for nested primers. The cDNA was amplified with 3′-RACE R2 and R3 primers and respective gene specific primers (GSP4: 5′-GGC TTT GTT TAC GGT CTG TC-3′ and GSP5: 5′-GTC TGT TTG GTT CAC TTT CC-3′). Amplified PCR products (5′ and 3′ RACE) were cloned, sequenced and analyzed.

The PCR (50 μl) reaction for the isolation of full length and error free *SbUSP* gene contained template cDNA (1 μg), dNTPs (200 μM), gene specific primers (0.2 μM each; F: 5′-ATG GCA ATG TCT GAT AAA CC-3′ & R: 5′-TCA GTG CTT AAT CTT AGG CTT-3′) and *Pfu* + *Taq* DNA polymerase (2:1). PCR was carried out and amplified products were purified, qualitatively analyzed on 1.0% agarose gels, cloned into the pGEM-*T* easy vector (Promega, USA) and sequenced at M/s Macrogen, S. Korea.

2.2. In-silico analysis

The *SbUSP* gene and *Sb*USP protein were characterized *in-silico* using computational biology. Amino acid sequences deduced from nucleotide sequences were imported into the ProtParam tool on ExPASy server (Gasteiger et al., 2005) for primary analysis and the PSIPRED server (Buchan et al., 2010) for secondary structure prediction and analysis. *Sb*USP amino acid sequences were subjected to BLAST and compared with the Protein Data Bank (PDB) and Conserved Domain Data bank (CCD) (Marchler-Bauer et al., 2013). Reference sequences which showed close similarity were retrieved and the phylogram analysis done using the USP sequence of the metazoan *Schistosoma* as out group. The phylogram was inferred by using the Maximum Likelihood (ML) statistical method based on the JTT matrix-based model using Molecular Evolutionary Genetics Analysis version 5 [MEGA5] MEGA5 (Tamura et al., 2011). The PDB database was explored for the comparative study of the USP protein domain and residue interactions with homologous domains using the online Cn3D4.1 software.

2.3. Transcript profiling of SbUSP gene under different stress

One month old seedlings were transferred to a hydroponic system containing 1/4 MS basal medium and grown under controlled laboratory conditions with 8/16 h dark/light cycle at 25 °C for 15 days. Plants were treated with different NaCl concentrations (0.05, 0.1, 0.25, 0.5 and 1.0 M) for 24 h. In a second set of experiments, seedling were subjected to different abiotic stresses (salt, 250 mM; drought; heat, 45 °C and cold, 4 °C) for different time periods (2, 6, 12, 24 h).

Total RNA was isolated from control and stressed (salt, drought, heat and cold) plant samples using the GITC method (Chomczynski and Sacchi, 1987), quantified using a Nanodrop spectrophotometer (NanoDrop, USA) and cDNA prepared using the Improm II reverse transcriptase first strand cDNA synthesis kit (Promega, USA). The Real Time qPCR (qRT PCR) reactions were carried out in 1× PCR buffer, 200 μM dNTPs, 1.25 U *Taq* DNA polymerase, 5 μM each gene specific primers (forward: 5′-GTT GGT TGT GGG TAG TCA-3′and reverse: 5′-AGG AGG ACA ATG CGT GCT T-3′ with primer binding site 63 and189 respectively) or β-tubulin primers (forward: 5′-GGA GTC ACC GAG GCA GAG-3′ and reverse: 5′-ATC ACA TAT CAG AAA CCA CAA AGC-3′) and 1× SYBR Green with PCR conditions of 95 °C—10 s, 60 °C—30 s and 72 °C—30 s for 35 cycles on a Bio-Rad thermal cycler and IQ5 detection system (Bio-Rad, USA). After PCR, products underwent melting curve analysis to check the specificity of PCR amplification in qRT PCR and amplified products were run on 1% agarose gels to confirm size. The qRT PCR data were analyzed by the comparative C_T method and relative fold gene expression ($2^{-\Delta\Delta C_T}$) of *SbUSP* in stressed plants compared to control (without treatment) was obtained by normalizing to β-tubulin (internal control) C_T values (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008).

2.4. Expression, purification and analysis of SbUSP–GST fusion protein

The complete coding region of the *SbUSP* gene were amplified using primers; F: 5′-GTC GAC ATG GCA ATG TCT GAT AAA CC-3′ (*Sal*I site underlined) and R: 5′-CTC GAG TCA GTG CTT AAT CTT AGG CTT-3′ (*Xho*I site underlined) and *Pfu* + *Taq* DNA polymerase. PCR products were ligated into the pGEX-6p-3 vector (Amersham Pharmacia Biotech, UK) treated with respective restriction enzymes. *E. coli* BL 21 (DE3) cells were transformed with the recombinant pGEX-USP vector and pGEX-6p-3 vector alone. The cloning and "framing" were confirmed by DNA sequencing. Recombinant protein was induced by treatment with IPTG (1 mM) for 6 h at 37 °C, purified under native conditions using glutathione-Sepharose 4B affinity columns (GE Healthcare, UK), quantified using the Bradford method, and analyzed by SDS-PAGE and western blotting. The GST and recombinant *Sb*USP–GST protein were isolated, electrophoresed (SDS-PAGE) and electroblotted to the PVDF membrane. Bound proteins of interest were detected by incubating with specific primary antibodies (1:1000 dilution; anti-GST antisera) and then subjected to secondary antibody (1:10,000 dilution) conjugated with