Characterization of the BETA1 gene, which might play a role in Beta vulgaris subsp. maritima salt tolerance

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Abstract: Salinity stress has a negative impact on plant growth, which affects homeostasis and productivity. The uptake of nonessential salt ions changes the osmotic balance of the cell and causes dehydration. Higher plants develop salt tolerance mechanisms to avoid dehydration. Sea beet (Beta vulgaris subsp. maritima) is a halophytic ancestor of cultivated sugar beet that displays salt stress tolerance. In this study, we screened a B. vulgaris subsp. maritima cDNA library in Saccharomyces cerevisiae strain Ab11c (ena1Δ, nha1/4Δ, nhx1Δ), which is deficient in sodium transport, to find sodium-detoxifying genes. We identified a cDNA construct, named BETA1, providing salt tolerance to yeast cells. This gene had no previously described function. Intracellular sodium measurements demonstrated no significant differences between yeast cells expressing BETA1 or a sham vector, suggesting that sodium was not effluxed in BETA1-expressing cells. Transcriptionally, BETA1 mRNA levels were induced immediately in leaves and later in the root system in response to the salt stress. Our results suggest that the BETA1 gene is part of the salt tolerance network in B. vulgaris subsp. maritima.

Key words: Salt stress, yeast, BETA1, Beta vulgaris subsp. maritima

1. Introduction

Plants are often exposed to salinity, drought, low and high temperatures, and other external abiotic stresses. Soil salinity is an important agricultural problem and United Nations Environment Programme researchers have shown that nearly 20% of agricultural and 50% of crop lands are affected by salt stress (Rubio et al., 1995). Salt stress is one of the most important abiotic stresses in plants and affects plant growth and productivity (Boyer, 1982; Zhu, 2002). However, some plants develop mechanisms to cope with salt stress. These mechanisms include ion homeostasis, osmotic adjustment, ion exclusion, deep rooting, stomatal closure, intracellular chelation, and increased antioxidant capacity (Flowers et al., 2005; Mickelbart et al., 2015). Salinity tolerance is a quantitative trait that is controlled by several genes like dreb1/cbf, dreb2, and areb/abf (Chinnusamy et al., 2005; RoyChoudhury et al., 2008; Fujita et al., 2011; Mizoi et al., 2012; Fujita et al., 2013).

Plants are categorized according to their responses to salinity. While salt-sensitive plants are affected even at low salt concentrations, salt-tolerant plants can absorb water from saline soil. Salt-sensitive plants can be classified as sensitive and moderately sensitive to soil salinity (Glenn et al., 1999; Chinnusamy et al., 2006). Salt-tolerant plants are known as halophytes (Parida and Das, 2005). Halophytes can survive at high salt conditions and evolved from glycophytes under salt stress condition (Winicov, 1998; Zhu, 2000).

Sea beet (Beta vulgaris subsp. maritima) is a wild relative of sugar beet (Lange et al., 1999). Sea beet grows in coastal and dry areas and can tolerate high concentrations of salt (Srivastava et al., 2000). Salt tolerance comparisons between sugar beet cultivars and their ancestor, sea beet, showed that sea beet had a higher salt tolerance than the sugar beet cultivars (Rozema et al., 2015). The salt tolerance mechanism of B. vulgaris subsp. maritima was previously studied. Bor et al. (2003) reported that B. vulgaris subsp. maritima exhibited a better protection mechanism against oxidative damage by maintaining higher inherited and induced activity of antioxidant enzymes than sugar beet (Bor et al., 2003). Transcriptomic profiling of the salt stress response of sea beet has been also investigated and several differentially expressed genes were identified (Skorupa et al., 2016).

To identify additional salt tolerance genes in sea beet, we used a functional genomic approach and screened a previously established cDNA library of B. vulgaris subsp. maritima in sodium transport-deficient yeast cells. After

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To identify additional salt tolerance genes in sea beet, we used a functional genomic approach and screened a previously established cDNA library of B. vulgaris subsp. maritima in sodium transport-deficient yeast cells. After
screening this library, we found a cDNA that confers salt tolerance to cells. This cDNA had no previously described function and we named it BETA1.

2. Materials and methods

2.1. Isolation and sequence analyses of the BETA1 and SAH7 genes

The wild-type yeast strain W303-1A (MATa; his3; leu2; met15; ura3) and its isogenic haploid Na⁺ transporter-deficient mutant Ab11c (ena1Δ, nha1/4Δ, nhx1Δ) was obtained from Hana Sychrova (Academy of Sciences of the Czech Republic, Department of Bioenergetics, Prague, Czech Republic).

The Beta vulgaris subsp. maritima cDNA library was transformed into Ab11c (ena1Δ, nha1/4Δ, nhx1Δ) yeast cells and screened on YNB plates containing 800 mM NaCl (Bozdağ et al., 2014).

Cloning of the BETA1 cDNA was performed using Gateway technology (Invitrogen, Karlsruhe, Germany). For expression purposes, the pAG426GPD-ccdB (Addgene, USA) overexpression vector was used. Sequence similarity searches were performed via the BLAST service of NCBI, and for multiple protein sequence alignments, T-coffee 6.85 software (Notredame et al., 2000) was used. The phylogenetic tree was calculated via MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) and the Newick format result of the tree was given to TreeDyn 198.3 (Chevenet et al., 2006) for tree rendering. Primers used for the amplification of SAH7 cDNA were AtSAH7F5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCTAAAGCAGTTCTATTGGTCG-3’ and AtSAH7R5’-GGGACACCATTCTTGTACAGAAAGCTGGGTCTGATGCTCGTTCGTTGTGATAGC-3’.

2.2. Yeast growth and media

Yeast transformation was performed using the standard lithium acetate method (Kaiser, 1994). Yeast cells were grown in either YPD medium (with 2% glucose, 2% peptone, 1% yeast extract, and 2% agar) or YNB (yeast nitrogen base) minimal medium including the required amino acids and bases. For the solid medium growth assays, wild-type yeast cells were transformed with either empty vector pAG426 and AB11c-pAG426 (control) cells were detected by different salts: LiCl (40 mM), KCl (1800 mM), and were diluted to OD600 = 0.2, 0.02, 0.002, or 0.0002 with distilled water. Next, 5 µL of each dilution was spotted onto solid SD plates with/without 800 mM NaCl, and were watered only with half-strength Hoagland solution. Control groups were watered only with half-strength Hoagland solution. Leaf and root samples were taken at 1, 3, 5, and 7 h after the addition of NaCl.

Total RNA isolation was performed using the Invitrogen RNA Isolation Kit (Invitrogen, Germany). DNase-treated total RNAs were used for cDNA synthesis using a cDNA Synthesis Kit (Fermentas, Sankt Leon-Rot, Germany). Maxima SYBR Green qPCR Master Mix was used (Fermentas, Germany) to perform the quantitative expression analyses of cDNAs using an iQ5 real-time PCR cycler system (Bio-Rad, Germany). The real-time PCR primers used for amplification of BETA1 cDNA were BETA1RTF 5’-TCTAGACCAAGAAAGACCTTAC-3’ and BETA1RTR5’-GGCACTTTCAACTTCACCTTTAC-3’, which were concurrently used to amplify 130 bp cDNA. Real-time PCR primers for beta actin cDNA were BmActRTF 5’-AGACCTTCAATGTGGCTGCT-3’ and BmActRTR 5’-TTCTCTGAGATGCACGCACC-3’, and these amplified 187 bp of Beta vulgaris subsp. maritima beta-actin cDNA. Gene expression levels were normalized with respect to BmACT expression levels. Real-time PCR data analyses were performed with Bio-Rad iQ5 software using Pfaffl’s model (Pfaffl, 2001). The conditions for PCR amplification were as follows: 95 °C for 5 min and 40 cycles of 94 °C for 25 s, 55 °C for 25 s, and 72 °C for 30 s.

2.4. Salt tolerance assay

The identified gene was tested under stress provided by different salts: LiCl (40 mM), KCl (1800 mM), and NaCl (800 mM). The wild-type and mutant strains were transformed with empty vector pAG426GPD. Yeast cells were incubated overnight in a shaker (170 rpm) at 30 °C and were diluted to OD600 = 0.2, 0.02, 0.002, or 0.0002 with distilled water. Next, 5 µL of each dilution was spotted onto solid SD plates with/without 800 mM NaCl, 40 mM LiCl, and 1800 mM KCl. Plates were incubated at 30 °C for 5 days.

2.5. Measurement of NaCl concentration in yeast cells

After NaCl treatment, the salt concentrations in yeast cells were watered only in half-strength Hoagland solution. Control groups were watered only with half-strength Hoagland solution. Leaf and root samples were taken at 1, 3, 5, and 7 h after the addition of NaCl.
Germany). Extracts were studied by induced coupled plasma-mass spectrometry (ICP-MS).

3. Results
3.1. Identification and cloning of the BETA1 gene
We screened a cDNA library of *B. maritima* to identify plant genes that conferred salt tolerance to yeast cells. Transformed yeast cells were plated onto selective media containing 800 mM NaCl, which is a toxic level for wild-type cells. After streaking these colonies onto new salt-containing plates, we decided to continue with one candidate that had confirmed growth on a toxic level of salt.

Plasmids were recovered from these colonies and sequenced with vector-based primers. The isolated cDNA was named BETA1 (accession number: XP_010676978). The BETA1 open reading frame consists of 662 bp and is composed of 61 amino acids. The calculated molecular weight of this protein was 17,666.1 Da and its isoelectric point was 6.45. The Blast(X) program was used for homology search and the multiple sequence alignment result for BETA1 is given in Figure 1. The BETA1 protein had no known function and shared 57% amino acid similarity with the *Arabidopsis thaliana* Sah7 (NP_567338.1) protein.

3.2. Characterization of BETA1 activity
After isolating the BETA1 gene from the *B. vulgaris* subsp. *maritima* library, we tested its cDNA independently to make sure that it provided salt tolerance in yeast cells. A spotting assay was performed on solid YNB-Ura medium and BETA1 expression provided tolerance to 800 mM NaCl in Ab11c yeast cells (Figure 2).

Because homology results showed that BETA1 had a 57% amino acid identity with the *Arabidopsis thaliana* SAH7 gene, we wanted to determine if Sah7 also played a role in salt tolerance. Therefore, we cloned its cDNA from *Arabidopsis thaliana* and did a spotting assay with cells expressing SAH7. As seen in Figure 2, SAH7 also conferred salt tolerance to the cells.

Genes with roles in salt tolerance often provide cross-resistance to other types of salts. To understand the specificity of the BETA1 and SAH7 genes, a salt tolerance assay was performed using different concentrations of KCl, LiCl, and NaCl. SAH7 and BETA1 showed the same growth patterns in 1 M KCl, 40 mM LiCl, and 800 mM NaCl media, while control Ab11c cells (with the pAG426GPD vector) did not grow under the same conditions (Figure 3). These results suggested that BETA1 and SAH7 were not specific for NaCl and that these genes were able to protect cells from stress conditions caused by KCl and LiCl.

3.3. Expression of the BETA1 gene in Beta vulgaris subsp. maritima
To evaluate the native expression pattern of the BETA1 gene, a real-time PCR assay was performed. *Beta vulgaris* maritima plants were grown under 400 mM NaCl conditions. Leaves and roots were harvested before and after NaCl treatment. The BETA1 mRNA level showed a significant increase in leaves (2-fold) compared with the control group (Figure 4a), suggesting that BETA1 might have roles in salt stress tolerance in leaves.

In root samples, there were no significant changes at early time points; however, expression of BETA1 was upregulated at about 5 h of treatment (2.5-fold) (Figure 4b).

3.4. Intracellular sodium concentrations
The sodium concentration of the yeast cells overexpressing BETA1 was measured to understand whether the BETA1 protein had a role in pumping sodium out of the cells. We observed no significant differences between control cells (W303 strain) with the empty pAG426GPD-ccdB plasmid and mutant cells that overexpressed the BETA1 gene (Figure 5). This suggested that BETA1 has no role in Na+ export.

4. Discussion
We screened a cDNA library of *Beta vulgaris* subsp. *maritima* to identify salt tolerance genes in the mutant Ab11c strain of *Saccharomyces cerevisiae*. Based on the solid growth test results, the BETA1 gene conferred promising salt tolerance to yeast cells. According to sequence and homology analyses, the SAH7 gene in *Arabidopsis thaliana* was a close homolog of the BETA1 gene in *B. vulgaris* subsp. *maritima*. SAH7 was first isolated in pollen tubes with unknown function and was found to be overexpressed under some stress conditions such as salt and antioxidant stress (Winter et al., 2007). Expression of SAH7 after salt treatment of *Arabidopsis* leaves showed 1.5-fold upregulation after 1 h of salt treatment and increased gradually up to 2-fold at 12 h (Winter et al., 2007). The BETA1 gene also showed a similar expression pattern in leaves, suggesting that these two genes were affected by salt in the early stages of abiotic stress. Transcriptional induction of these genes takes longer in roots than in leaves (Winter et al., 2007). These results showed that BETA1 and SAH7 react quicker to salt stress in leaves than roots. Dunajskà-Ordak et al. (2014) reported that peroxisomal ascorbate peroxidase (BvpAPX) was upregulated upon prolonged salt stress in leaves of both wild and cultivated beets. However, a longer exposure to salinity was required to stimulate BvpAPX expression in salt-tolerant *B. vulgaris* subsp. *maritima* when compared with sugar beet varieties. Ghoulam et al. (2002) suggested that sea beet accumulates more salt in leaves than tap roots to increase turgor and adjust its osmotic value by accumulating osmolytes. It is likely that early gene expression of BETA1 and SAH7 in leaves is related to osmoregulation.
Figure 1. Multiple amino acid sequence alignment of BETA1 protein sequence. a) Colors indicate conserved amino acids between Beta vulgaris subsp. vulgaris (XP_010676978.1), Vitis pseudoreticulata (ABC86745.1), Vitis vinifera (XP_002634471.1), Vitis vinifera (CB119132.3), Vitis vinifera (XP_002268073.1), Vitis vinifera (CB13037.3), Cucumis melo (XP_008445353.1), Cucumis sativus (XP_004150698.1), Cucumis sativus (KGN62176.1), and Arabidopsis thaliana (NP_567338.1). b) Phylogenetic tree showing relationships among the proteins.
Figure 2. Salt tolerance conferred by BETA1 expression in yeast cells. W303-1A is the wild-type and Ab11c (ena1Δ, nha1/4Δ, nhx1Δ) is an isogenic sodium transporter-deficient yeast strain. Tenfold serial dilutions of cell cultures were spotted on YNB-Ura medium containing 800 mM NaCl. Plates were incubated at 30 °C for 5 days and photographed.

Figure 3. Growth of BETA1- and SAH7-expressing yeast cells in the presence of different alkali metal cations. Tenfold dilutions of cultures were spotted on YNB-Ura medium with shown concentrations of KCl, LiCl, and NaCl and plates were incubated at 30 °C for 5 days.

Figure 4. Expression analyses of BETA1 mRNA at different time points after salt treatment: (a) expression patterns in leaves, (b) expression patterns in roots. Plants were exposed to 400 mM NaCl and values are the means of three replicates. Error bars represent the standard error of the mean (n = 3).
According our growth test results, BETA1 and SAH7 showed similar patterns. Both grew on medium with toxic levels of KCl, LiCl, and NaCl. These results suggested that BETA1 and SAH7 were not substrate-specific and that these genes were able to protect cells from stress conditions caused by alkali metal cations.

The Sah7 protein is localized in the ER, extracellular Golgi, and nucleus (Tanz et al., 2013; Hooper et al., 2014). In subcellular localization prediction results, Beta1 protein was localized to the endomembrane system of the cell. Endomembrane localized N-glycosylation proteins are known to regulate salt tolerance, cellulose biosynthesis, and protein quality control in plants (Kang et al., 2008). In the Golgi apparatus, N-glycan maturation confers salt sensitivity (Kang et al., 2008; von Schaewen et al., 2008). Several posttranslational modifications occur in the endomembrane system in the cell; therefore, BETA1 and SAH7 might have a role in this pathway.

Sea beet has the ability to endure salt stress resistance due to efficient osmotic adjustment, which is generated by accumulation of more Na⁺ and Cl⁻ in the leaves and sucrose as well as proline in tap roots (Ghoulam et al., 2002; Bagatta et al., 2008). Intracellular Na⁺ concentration measurements revealed no differences between cells carrying BETA1 and the sham vector. BETA1 may play a role in the transport of excess Na⁺ into the endomembrane system to decrease the concentration of toxic Na⁺ in the cytoplasm. Thus, BETA1 might help the cell to sequester sodium into the endomembrane system.

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References


