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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 (*ena1*Δ, *nha1*/4Δ, *nhx1*Δ), 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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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 (*ena1*Δ, *nha1*/4Δ, *nhx1*Δ) 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 (*ena1*Δ, *nha1*/4Δ, *nhx1*Δ) yeast cells and screened on YNB plates containing 800 mM NaCl (Bozdag 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'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAT GTCTAAAGCAGTTCTATTGGTCG-3' and AtSAH7R 5'-GGGGACCACTTTGTAC AAGAAAGCTGGGTC CTAGTCCTCGGTTTCTTGGTATAGC-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 pAG426GPD or *BETA1*/*SAH7* cDNA containing the pAG426GPD overexpression vector. Yeast cells (shaken at 200 rpm, at 30 °C), after overnight incubation, were diluted to OD600 = 0.2, 0.02, 0.002, or 0.0002 with sterile distilled water, and 5 µL of these dilutions were transferred to spots on solid YNB (–ura; 2% glucose) plates without NaCl or with 800 mM NaCl. Cells were incubated for 5 days at 30 °C and plates were photographed.

2.3. Plant growth, RNA isolation, and real-time PCR analyses

B. vulgaris subsp. *maritima* plants were grown in half-strength Hoagland solution (Hoagland and Arnon, 1950) in a growth chamber under conditions of 12 h of dark and 12 h of light at 25 °C. Stress treatment was started when seedlings were 15 days old by the addition of 400 mM NaCl 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'-GTAGACCAGAGAAGAAGCCATAC-3' and BETA1RTR 5'- GGCATTCCAACTTCACCTTTAC-3', which were concurrently used to amplify 130 bp of cDNA. Real-time PCR primers for beta actin cDNA were BmActRTF 5'-AGACCTTCAATGTGCCTGCT-3' and BmActRTR 5'-TCAGTGAGATCACGACCAGC-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 *BETA1*-pAG426 and AB11c-pAG426 (control) cells were detected (Mizuno et al., 2005). Precultured yeast cells were grown overnight in SD medium and the cells were diluted with the SD medium to a final concentration of 1/1000 diluted yeast cells. NaCl (800 mM) was added and cells were incubated for 48–60 h at 30 °C. Cells were then washed with 10 mM EDTA three times and dried overnight at 70 °C. Yeast cells (5 mg) were extracted by 65% HNO_3 and filtrated with 0.20-µm Minisart filters (Gottingen,

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* subsp. *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.

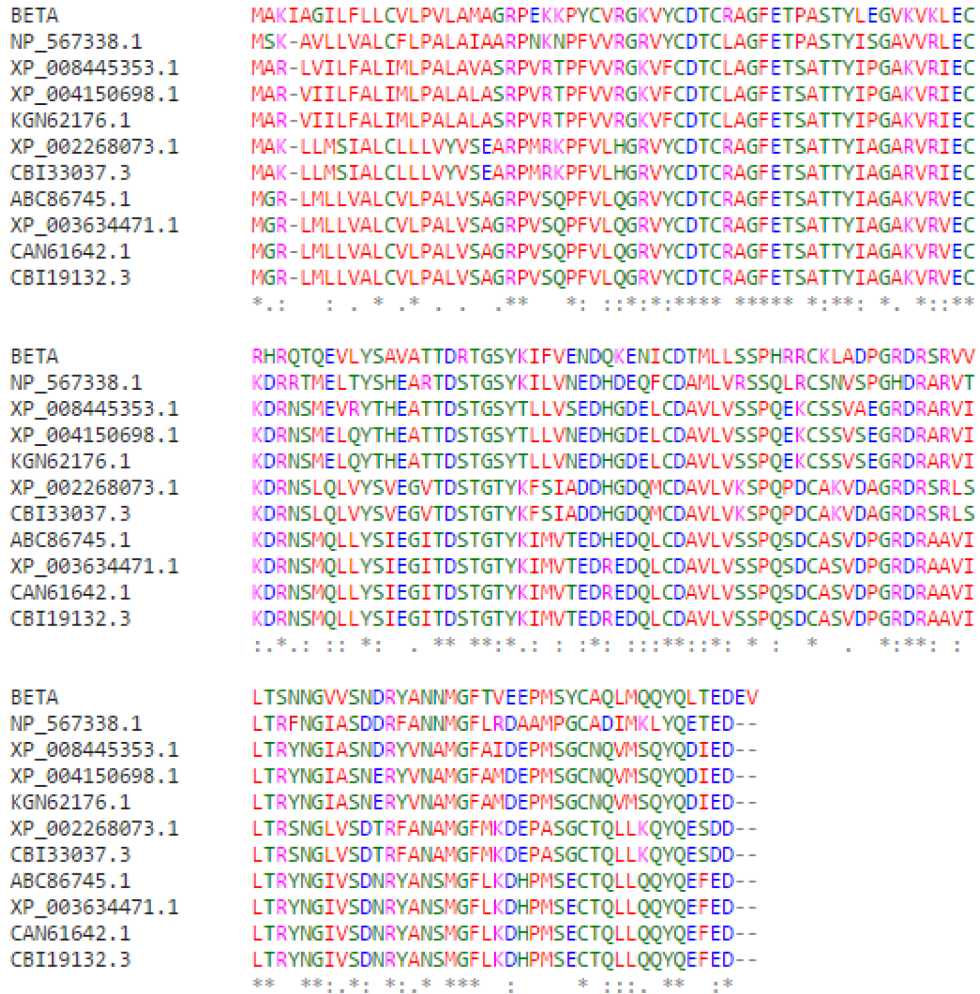
In root samples, there were no significant changes at early time points; however, expression of *BETA1* was upregulated at about 5 h of treatment (2.5-fold) (Figure 4b).

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. Dunajska-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.



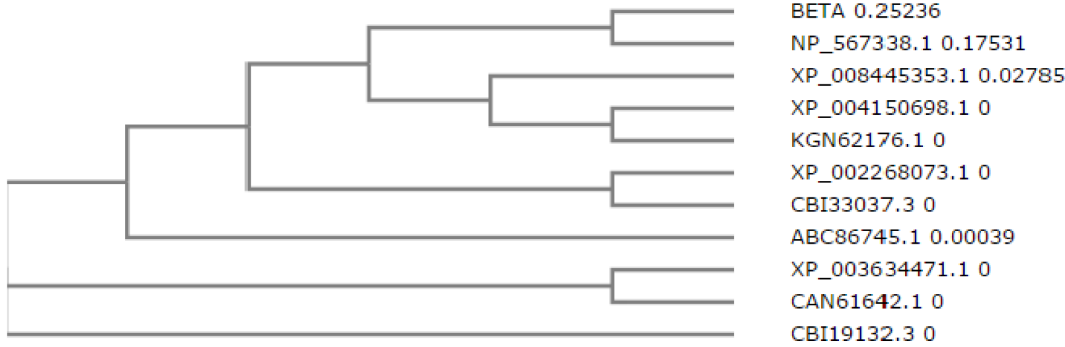


Figure 1. Multiple amino acid sequence alignment of *BETA1* protein sequence. a) Colors indicate conserved amino acids between *Beta vulgaris* subsp. *vulgaris* (XP_010676978.1) *Vitis pseudoreticulata* (ABC86745.1), *Vitis vinifera* (XP_003634471.1), *Vitis vinifera* (CBI19132.3), *Vitis vinifera* (XP_002268073.1), *Vitis vinifera* (CBI33037.3), *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.

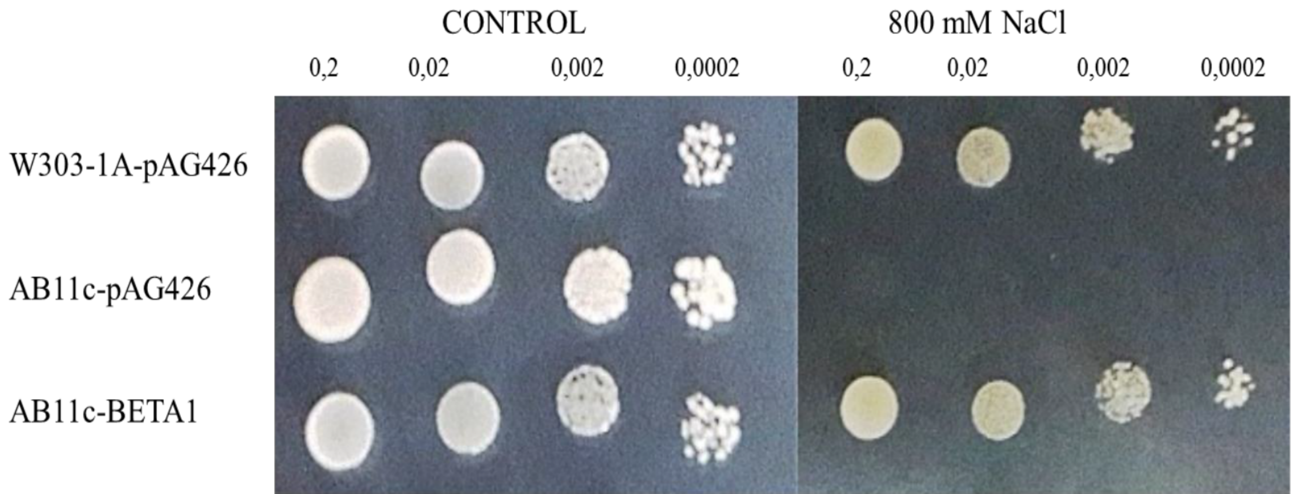


Figure 2. Salt tolerance conferred by *BETA1* expression in yeast cells. W303-1A is the wild-type and Ab11c (*ena1Δ*, *nha1/4Δ*, *nhx1Δ*) is an isogenic sodium transporter-deficient yeast strain. Tenfold serial dilutions of cell cultures were spotted on YNB-Ura medium containing 800 mM NaCl. Plates were incubated at 30 °C for 5 days and photographed.

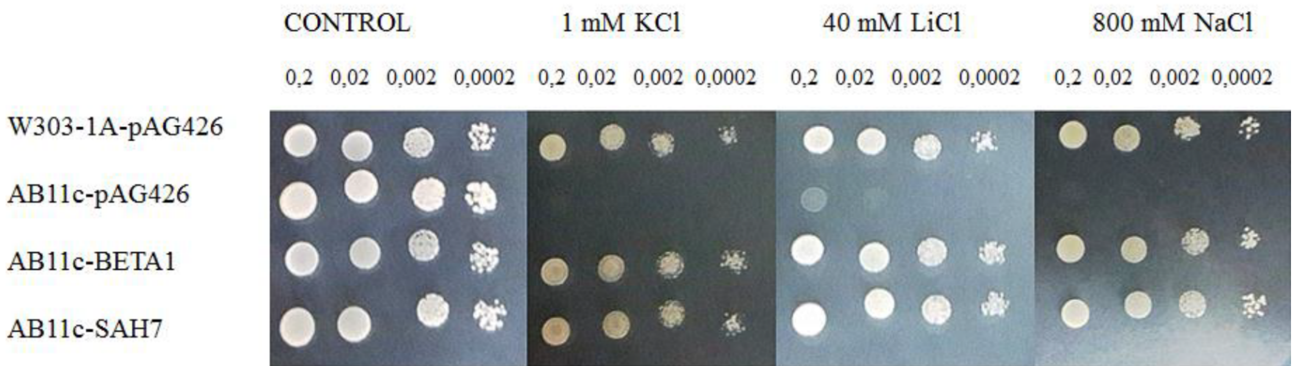


Figure 3. Growth of *BETA1*- and *SAH7*-expressing yeast cells in the presence of different alkali metal cations. Tenfold dilutions of cultures were spotted on YNB-Ura medium with shown concentrations of KCl, LiCl, and NaCl and plates were incubated at 30 °C for 5 days.

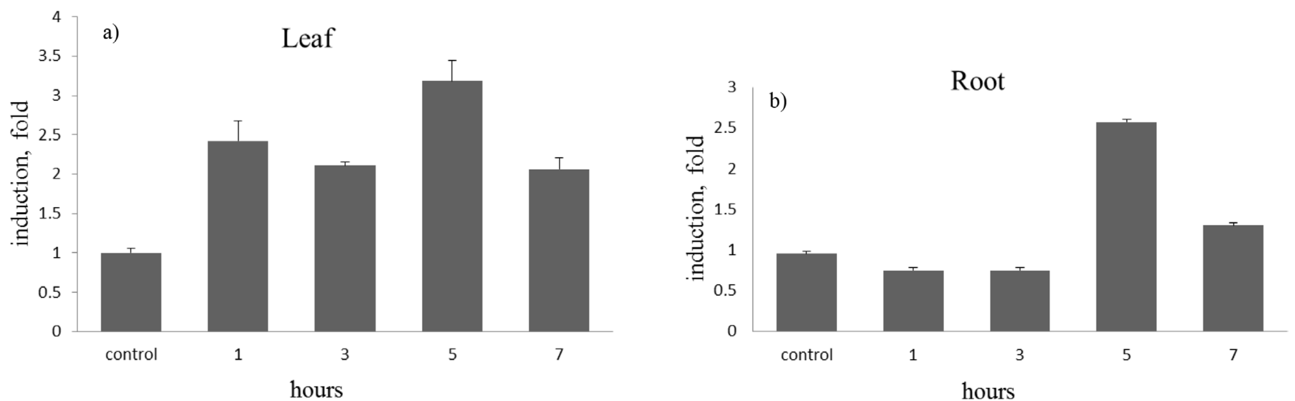


Figure 4. Expression analyses of *BETA1* mRNA at different time points after salt treatment: (a) expression patterns in leaves, (b) expression patterns in roots. Plants were exposed to 400 mM NaCl and values are the means of three replicates. Error bars represent the standard error of the mean (n = 3).

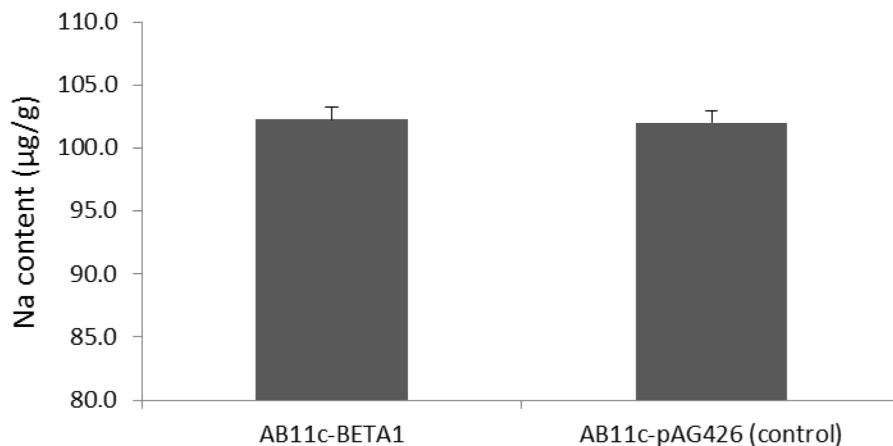


Figure 5. Intracellular sodium concentrations. Cells expressing the *BETA1* gene (BETA1-pAG426) did not have altered Na^+ levels. Control samples contained an empty vector (AB11c-pAG426). Values are the means of two independent experiments with 2 replicates (±SEM, n = 4). Error bars represent the standard error of the mean (n = 4).

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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