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Over-expression of a subunit E1 of a vacuolar H^+-ATPase gene (*Lm VHA-E1*) cloned from the halophyte *Lobularia maritima* improves the tolerance of *Arabidopsis thaliana* to salt and osmotic stresses

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ABSTRACT

In order to understand the molecular basis of vacuolar H^+-ATPase subunits and reveal their possible role in salt and drought tolerance in plant species, a vacuolar H^+-ATPase subunit E1 gene (*LmVHA-E1*) was isolated from the halophyte *Lobularia maritima* and over-expressed in the glycophyte *Arabidopsis thaliana*. Q-RT-PCR demonstrated that the expression of this gene was induced in *L. maritima* in response to salt stress and PEG-induced osmotic stress. The overexpression of *LmVHA-E1* in *Arabidopsis thaliana* conferred high salt and drought tolerance to transgenic plants. Transgenic *Arabidopsis* plants showed higher seedling survival rates and better antioxidant activities under salt and drought stress. The analysis of ion content and osmotic potentials indicated that under high salinity the transgenic plants compartmentalized more Na^+ and showed enhanced osmotic adjustment in the leaves compared with wild-type plants. Accordingly, the higher levels of expression of different stress related genes such as *AtNHX1 AtP5CS*, *AtCAT*, *AtSOD*, AtPOD and *AtLEA*, indicated higher levels of activities in sodium sequestration into vacuoles, in osmotic regulation and in ROS scavenging of transgenic plants.

1. Introduction

Depending on their growth performance in saline habitats, plants can be divided into salt sensitive glycophytes and salt tolerant halophytes. Halophytes, in contrast to glycophytes, can generally tolerate high salt concentrations and they can be defined as plants that can complete their whole life cycle at salt concentrations higher than 200 mM NaCl (Flowers and Colmer 2008). Both groups possess the same mechanisms of salt tolerance; it is the differential regulation of these mechanisms that makes the eventual difference (Flowers and Colmer 2008). In most of the instances, the difference in post translational regulation between halophytes and glycophytes causes the development of salt sensitive or salt-tolerant phenotypes (Edelist et al., 2009; Bonales-Alatorre et al., 2013a,b). Recently, the comparison of the H^+-ATPase transcript levels in both halophytes and glycophytes proved the importance of post-translational modifications in halophytes for enhanced salinity tolerance (Bose et al., 2015).

In order to improve drought and salt tolerance in crop plants, it is important to isolate candidate genes from halophytes that can be transferred to sensitive crop species (Himabindu et al., 2016; Ozfidan-Konakci et al., 2016). However, most halophyte species are not yet amenable for such kind of studies due to limited information on their gene sequences and the difficulty to identify salt-regulated genes and their products. In contrast, the model plants with complete sequencing of their genomes – *Arabidopsis* and rice – are typical glycophytes that are not very salt-tolerant. For comparative genomics studies, halophyte species would be most suitable that are closely related to the completely sequenced plant models to take advantage of the wealth of genomic data from these species. One ideal halophyte model that meets these criteria is *Lobularia maritima* L. Desv., commonly known by sweet alyssum – a species of the Brassicaceae that is distributed throughout the Mediterranean basin, where it grows in coastal zones, dunes and scrublands (Picó and Retana, 2001). Transcripts from *Lobularia* share in average 90% identity with homologous genes from *Arabidopsis* (Popova and Golldack, 2007; Popova et al., 2008).

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Lobularia does not possess morphological specializations such as bladder cells or salt glands. Tolerance of the species is due to adjustment in ion and osmotic homeostasis (Golldack 2004). Popova and Golldack (2007) reported that the salt adaptation of this halophyte was correlated with the activation of the vacuolar Na^+/H^+ antiporter and the vacuolar H^+-ATPase. They showed that the steady state transcript amounts of *VHA-E* increased in salt treated leaves of *L. maritima*.

Salt-induced expression of V-ATPase subunits has also been shown for other halophytes such as *Mesembryanthemum crystallinum* and *Suaeda salsa* (Ratajczak et al., 1994; Golldack and Deitz, 2000; Wang et al., 2001) as well as for salt-adapted suspension cells of tobacco and for salt-tolerant *Beta vulgaris* (Narasimhan et al., 1991; Kirsch et al., 1996; Lehr et al., 1999). In contrast, no salt-induced transcription of V-ATPase subunits has been observed for glycophytes, such as *A. thaliana* (Kluge et al., 1999) indicating salt-dependent activation of V-ATPase to be a specific response mechanism in halophytes that is missing in glycophytes.

In the current study, *VHA-E1* was isolated from the leaves of the halophyte *L. maritima* and transferred to the glycophyte *A. thaliana* in order to improve its tolerance to high salinity and drought stresses.

2. Materials and methods

2.1. Plant materials

Seeds of *L. maritima* were harvested in Borj Cedria, a locality close to the Mediterranean seashore, 20 km north of Tunis. *A. thaliana* (Columbia ecotype) were used in this study for genetic transformation.

2.2. Culture conditions and stress treatments

Seeds of *L. maritima* were germinated in pots filled with a mixture of sandy soil and organic matter. The soil was a limono-sandy soil, the pH and the electrical conductivity of the aqueous extract (1/10) were 6.65 and 0.05 mmhos cm^{-1} respectively. Seeds were watered with Milli-Q water until germination, and then irrigated with 10% Hoagland's nutrient solution (Epstein, 1972). The pots were placed in a greenhouse under controlled conditions (16 h photoperiod; 300 μmol m^{-2} s^{-1} PAR). The mean temperature and the relative humidity were respectively 30 ± 5 °C, 55 ± 5% during the day and 16 ± 2 °C, 90 ± 5% during the night.

After 4 weeks, different stress conditions were applied: Salinity (400 mM NaCl), water deficit stress (10% (w/v) PEG 8000). The fully expanded leaves were sampled after 6, 24 and 48 h of the beginning of each treatment, frozen in liquid nitrogen and stored at −80 °C for RNA extraction.

2.3. Extraction of RNA, synthesis of cDNA and isolation of LmVHA-E1 gene

Total RNA was isolated from *L. maritima* plants subjected to various stress treatments using the Trizol reagent (Invitrogen) according to the manufacturer's protocol. To remove the remaining genomic DNA, the RNA was treated with DNaseI (MBI, Fermantas) at 37 °C for 15 min. To generate the full-length cDNA of *LmVHA-E1*, DNase-treated RNA samples (5 μg) were reverse-transcribed using M-MLV reverse transcriptase (Invitrogen). The reverse transcription (RT) reactions were performed at 37 °C for 1 h using 2 μM oligo-dT18. Diluted aliquots (1:20) of the resulting RT-reaction product were then employed as a template for PCR amplification generating *LmVHA-E1* specific fragment using two specific primers *VHA-E1*-F and *VHA-E1*-R (supplementary Table 1). The reaction included an initial 3 min of denaturation at 94 °C, then 35 cycles of 30 s at 94 °C, 45 s at 55 °C and 1 min at 72 °C, and finally 10 min extension at 72 °C. The resulting fragment was cloned in the pGEMT-Easy vector (Promega) and three different positive clones were sequenced using SP6 and T7 primers.

2.4. Real-time quantitative PCR

The transcription levels of the *LmVHA-E1* gene in *L. maritima* plants subjected to NaCl and PEG were assessed by real-time qPCR. QPCR was also used to measure the transcript accumulation of six stress-related genes in *LmVHA-E1* transgenic lines and WT *Arabidopsis* plants grown under control, salt (150 mM NaCl) and osmotic (200 mM mannitol) stress conditions. These genes encode: Na^+ transporter (*AtNHX1*, At5G27150), pyrroline-5-carboxylatesynthetase (*AtP5CS*, At3G55610), catalase (*AtCAT*, AtNM101914), manganese superoxide dismutase (*AtMn-SOD*, At3G56350), peroxidase (*AtPOD*, At3G49120), and LEA proteins (*AtLEA*, At1G02820). Primer pairs were designed with Primer 3 software to ensure gene specificity in amplification of the *LmVHA-E1* gene, six stress-related genes and the house-keeping Ubiquitin 10 mRNA (UBQ10: *At*4g05320) gene (Gruber et al., 2001; Rozen and Skaletsky 2000). The amplification of genes was performed using following gene-specific primers: *VHA-E1* (*LmVHA-EF, LmVHA-E1R*), *NHX1* (*AtNHX1F, AtNHX1R*), *P5CS* (*AtP5CSF, AtP5CSR*), *CAT* (*AtCATF, AtCATR*), *SOD* (*AtMn-SODF, AtMn-SODR*), *POD* (*AtPODF, AtPODR*) and *LEA* (*AtLEAF, AtLEAR*) (Supplementary Table 1).

Total RNA was extracted from WT *Arabidopsis* and transgenic lines and then reverse-transcribed using oligo-dT (18 mer) as described above to generate first strand cDNA. The RT reactions were diluted 1:5 and used as template in real-time qPCR reactions. The amplification reactions were performed in 15 μl final volumes containing 7.5 μl 2 x Quantitect SYBR Green I mixture (Qiagen), 1.5 μl primer-pair mix (0.5/0.5 μM for forward and reverse primers), 3 μl cDNA, and 3 μl RNase-free water. Reactions were carried out in a Light-Cycler 480 (Roche, Basel, Switzerland). Thermal cycling conditions were 5 min at 95 °C, followed by 45 cycles of 20 s at 95 °C, 15 s at 60 °C, and 20 s at 72 °C. Melting curve analysis at the end of cycling was used to verify that was single amplification. At the end of the reaction, the threshold cycle (C_T) values of the triplicate PCRs were averaged and used for transcripts quantification. The relative expression ratio of *LmVHA-E1* gene and the six stress-related genes was calculated by using the comparative C_T method with the UBQ10 gene as an internal expression standard (Livak and Schmittgen 2001). The relative expression level was calculated from triplicate measurements based on the $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = (CT, _{Targetgene} - CT, _{UBQ10})$ stressed − $(CT, _{Targetgene} - CT, _{UBQ10})$ control. Relative expression ratios from three independent experiments (three biological repetitions) are reported.

2.5. Gene transfer in Arabidopsis thaliana

To produce transgenic *A. thaliana* plants harbouring *LmVHA-E1* cDNA, the pGEMT-Easy was digested with *Nco*I/*Spe*I to release the cDNA fragment of *LmVHA-E1* gene which was purified of the gel and finally cloned in the binary vector pCAMBIA 1380 (CAMBIA, Canberra, Australia) under the control of the CaMV35S promoter and CaMV35S terminator. The obtained construct pCAMBIA 1380-*LmVHA-E1* was then mobilized into *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al., 1983) by freeze-thaw transformation method (Chen et al., 1994). The recombinant *Agrobacterium* was thereafter used for *A. thaliana* transformation by the floral dip technique (Clough and Bent 1998). Transgenic plants were selected on MS agar medium (Murashige and Skoog 1962) containing 20 μg/l hygromycin. Seeds of the T2 generation were harvested and the seedlings from the homozygous T3 generation were used. The