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Expression of wheat Na^+/H^+ antiporter *TNHXS1* and H^+ - pyrophosphatase *TVP1* genes in tobacco from a bicistronic transcriptional unit improves salt tolerance

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Abstract

Abiotic stress tolerance of plants is a very complex trait and involves multiple physiological and biochemical processes. Thus, the improvement of plant stress tolerance should involve pyramiding of multiple genes. In the present study, we report the construction and application of a bicistronic system, involving the internal ribosome entry site (IRES) sequence from the 5'UTR of the heat-shock protein of tobacco gene *NtHSF-1*, to the improvement of salt tolerance in transgenic tobacco plants. Two genes from wheat encoding two important vacuolar ion transporters, Na⁺/H⁺ antiporter (*TNHXS1*) and H⁺-pyrophosphatase (*TVPI*), were linked via IRES to generate the bicistronic construct *TNHXS1*-IRES-*TVPI*. Molecular analysis of transgenic tobacco plants revealed the correct integration of the *TNHXS1*-IRES-*TVPI* construct into tobacco genome and the production of the full-length bicistronic mRNA from the 35S promoter. Transgenic tobacco plants expressing *TNHXS1*-IRES-*TVPI* showed a better performance than either of the single gene-transformed lines and the wild type plants when subjected to salt treatment. Transgenic *TNHXS1*-IRES-*TVPI* plants exhibited a well-developed root system, greater biomass production, less chlorophyll loss, lower membrane damage, higher antioxidant capacity, and higher relative water content. Interestingly, the *TNHXS1*-IRES-*TVPI* transgenic plants accumulated less Na⁺ and more K⁺ in their leaf tissue than did the wild type and the single gene-transformed lines. These results demonstrate that IRES system, described herein, can co-ordinate the expression of two important abiotic stress-tolerance genes and that this expression system is a valuable tool for obtaining transgenic plants with improved salt tolerance.

1 **Expression of wheat Na^+/H^+ antiporter *TNHXS1* and H^+ - pyrophosphatase *TVPI***
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Abstract

Abiotic stress tolerance of plants is a very complex trait and involves multiple physiological and biochemical processes. Thus, the improvement of plant stress tolerance should involve pyramiding of multiple genes. In the present study, we report the construction and application of a bicistronic system, involving the internal ribosome entry site (IRES) sequence from the 5'UTR of the heat-shock protein of tobacco gene *NtHSF-1*, to the improvement of salt tolerance in transgenic tobacco plants. Two genes from wheat encoding two important vacuolar ion transporters, Na⁺/H⁺ antiporter (*TNHXS1*) and H⁺-pyrophosphatase (*TVPI*), were linked via IRES to generate the bicistronic construct *TNHXS1*-IRES-*TVPI*. Molecular analysis of transgenic tobacco plants revealed the correct integration of the *TNHXS1*-IRES-*TVPI* construct into tobacco genome and the production of the full-length bicistronic mRNA from the 35S promoter. Transgenic tobacco plants expressing *TNHXS1*-IRES-*TVPI* showed a better performance than either of the single gene-transformed lines and the wild type plants when subjected to salt treatment. Transgenic *TNHXS1*-IRES-*TVPI* plants exhibited a well-developed root system, greater biomass production, less chlorophyll loss, lower membrane damage, higher antioxidant capacity, and higher relative water content. Interestingly, the *TNHXS1*-IRES-*TVPI* transgenic plants accumulated less Na⁺ and more K⁺ in their leaf tissue than did the wild type and the single gene-transformed lines. These results demonstrate that IRES system, described herein, can co-ordinate the expression of two important abiotic stress-tolerance genes and that this expression system is a valuable tool for obtaining transgenic plants with improved salt tolerance.

Keywords: Bicistronic. Gene pyramiding. H⁺-PPase. IRES. *TaNHXS1*. Salt tolerance.

1 Introduction

2
3 Salinity is one of the most important environmental constraints affecting more than 800 million ha of
4 arable land (Munns and Tester, 2008). Salinity limits plant growth and causes significant losses of crop
5 yield. Plants have evolved several mechanisms to cope with salt stress, the most important of which are
6 Na^+ exclusion from the shoot, Na^+ tissue tolerance, and osmotic tolerance. One key mechanism of
7 salinity tolerance is the ability to remove Na^+ ions from the cytosol and its sequestration into the
8 vacuole to limit cell damage. The transport of Na^+ into vacuoles is thought to be mediated by vacuolar
9 Na^+/H^+ antiporters of the NHX family, which are driven by the electrochemical gradient of protons
10 (Gaxiola et al. 1999; Pardo et al. 2006). The proton motive force used by vacuolar antiporters is
11 generated by the H^+ -translocating enzymes in the tonoplast, which are V-ATPase and H^+ -inorganic
12 pyrophosphatase (H^+ -PPiase). The H^+ -PPiase is a single subunit protein located in the vacuolar
13 membrane (Maeshima, 2000). It pumps H^+ from the cytoplasm into vacuoles with PPi-dependant H^+
14 transport activity. Zhen et al. (1997) demonstrated that the Arabidopsis *AtAVPI* generated a proton
15 gradient across the vacuolar membrane similar in magnitude to that of the vacuolar H^+ -ATPase.

16 The over-expression of *AtNHX1*, or its homologs from other plant species, has resulted in
17 enhanced plant tolerance not only to salt, but also to drought stress (Apse et al. 1999; Zhang and
18 Blumwald, 2001; Xue et al. 2004; He et al. 2005; Brini et al. 2007; Chen et al. 2007; Khoudi et al.
19 2009; Liu et al. 2010). Similarly, the over-expression of *AtAVPI* or its homologs in transgenic plants
20 resulted in both drought and salt tolerance (Gaxiola et al. 2001). Interestingly, the enhanced salt
21 tolerance in transgenic plants, over-expressing the *AtAVPI* gene, was correlated with the increased ion
22 content. These results suggest that the enhanced vacuolar H^+ pumping in transgenic plants was
23 responsible for additional energy to support vacuolar sodium accumulation mediated by the Na^+/H^+
24 antiporter. In line with these results, Duan et al. (2007) demonstrated that the over-expression of H^+ -
25 PPase resulted in an enhanced proton electrochemical gradient across the vacuolar membrane which
26 accelerated the sequestration of Na^+ into the vacuole and improved salinity tolerance at cellular level.
27 Thus, it was predicted that co-expression of Na^+/H^+ antiporter and H^+ -pyrophosphatase would confer
28 better salt tolerance to transgenic plants than that of the single gene expression. Indeed, Zhao et al.
29 (2006) provided experimental evidence that co-expression of *SsNHX1* and *AtAVPI* in transgenic rice
30 conferred greater salt tolerance than that of the single *SsNHX1*. On the other hand, Qiu et al. (2007)

1 reported that the coordinate expression of V-ATPase and vacuolar Na⁺/H⁺ antiporter was essential for
2 *Suaeda salsa* salt tolerance.

3 Salt stress tolerance is a complex trait (Flowers, 2004); therefore, better tolerance to salinity
4 might be achieved by pyramiding several genes into a single genotype (Samis et al. 2001). The success
5 of this approach was demonstrated in the above-mentioned examples for pyramiding Na⁺/H⁺ antiporters
6 and one of the vacuolar translocating enzymes (H⁺-PPase or V-H⁺ATPase). It was also used for
7 stacking various combinations of genes involved in drought and salt stress tolerance (Singla-Pareek et
8 al. 2003; Zhou et al. 2008; Wei et al. 2011). To engineer plants with multiple genes, Schmidt et al.
9 (2008) described several approaches. For example, plants are individually transformed and then crossed
10 together. This method complicates the downstream breeding due to the size of the segregation
11 population needed to obtain all the transgenes in one individual. An alternative approach consists of
12 performing repeated plant transformation with successive transgenes to obtain multi-gene phenotypes.
13 However, this approach is costly, time consuming, and it needs several selectable marker genes to
14 perform the various selection rounds. One practical solution to overcome these limitations is to
15 physically link multiple gene expression cassettes by positioning them contiguously on DNA that will
16 transfer as single entity (Shibata and Liu, 2000). This strategy has been used with great success to stack
17 traits in commercial GM crops.

18 In the present study, transgenic tobacco plants were produced by the introduction of a
19 dicistronic construct of wheat vacuolar Na⁺/H⁺ antiporter and H⁺-pyrophosphatase genes. These plants
20 were used to investigate whether co-ordinating the expression of these two genes, using an IRES
21 system, would result in higher salinity tolerance than plants with a single transgene.

22 23 **Materials and methods**

24 25 Cloning of the vacuolar Na⁺/H⁺ antiporter (*TNHXS1*) and sequence analysis

26
27 Based on the sequence of the vacuolar antiporter reported in Brini et al. (2005), we designed two
28 specific primers, TN.1: 5' CATTAGCTGCAGATGGGGCTCGATTTGGGA and TN.2: 5'-
29 CTAATGTCTAGAACTACGGTCTTCTGCCTC-3'. Two restriction sites were included in the primer
30 sequences to allow directional cloning in the expression cassettes: *Pst*I site for TN.1 and *Xba*I for TN.2.

1 These two primers were used to PCR-amplify the full-length *TNHXS1* ORF from a cDNA library
2 constructed from root tissue of bread wheat. PCR products were purified from agarose gel, cloned in
3 pGEM-T easy vector and sequenced (double-strand) using ABI PRISM automated sequencer. Blast
4 tool was used to check for similarity with other NHXs in the data bases online
5 (<http://www.ncbi.nlm.nih.gov>).

6 7 Yeast transformation, cation tolerance test and intracellular ion content determination

8
9 For functional complementation analysis, *TNHXS1* cDNA was subcloned into the pYES2 yeast
10 expression vector. The plasmid was transformed into the yeast strain AXT3 ($\Delta ena1::HIS3::ena4$
11 $nah1::LEU2 nhx1::KanMX ura3-1 trp1 ade2-1 can1-100$) (Quintero et al., 2002) using the standard
12 polyethylene glycol (PEG) lithium acetate method (Elble 1992). For cation tolerance test, saturated
13 yeast cultures were diluted to an absorbance of 0.8 at 600 nm with YPG solution (1% yeast extract, 2%
14 peptone, 0.01 % glucose, 2 % galactose). Aliquots (5 μ l) of the OD₆₀₀ = 0.8 yeast cultures or 10-fold
15 serial dilutions were spotted onto different YPG plates and grown at 30 °C for 5 days. In addition, a 10
16 μ l OD₆₀₀ = 0.8 culture was inoculated into 10 ml YPG, with or without 100 mM NaCl or 1 M KCl and
17 the absorbance at 600 nm was measured after shaking at 30 °C for 48 h.

18 The total Na⁺ and K⁺ content of the different yeast cells grown in YPG supplemented with 100 mM
19 NaCl or 1 M KCl was determined as described in Teakle et al. (2010). The yeast cells were collected
20 by centrifugation 5 min at 2000g and then washed twice with ice-cold (1.2 M Iso-osmotic sorbitol, 20
21 mM MgSO₄). Intracellular ions were extracted by HNO₃ (0.5 M) digestion and analysed by atomic
22 absorption spectrometry.

23 24 Cloning of internal ribosome entry site (IRES) element

25
26 We designed two specific primers based on nucleotide sequence of the *Nicotiana tabacum* heat-shock
27 factor1 (*NtHSF-1*) mRNA (EMBL accession no. AB014483), with sequences SP.1: (5'-
28 CATTAGTCTAGAGGCACGAGGCTCCCATTAAT-3' and SP.2: 5'-
29 CTAATGGGATCCCCCTTGT TTTTCCCCTGT-3'. Two restriction sites were included in the primer
30 sequences to allow cloning of the IRES in the expression cassette. These two sites are *Bam*HI and *Xba*I
31 for SP.1 and SP.2, respectively. SP.1 and SP.2 were used to PCR-amplify the IRES sequence using

total tobacco genomic DNA. PCR products were gel purified, cloned in pGEM-T easy vector and sequenced (double-strand) using ABI PRISM automated sequencer.

Cloning of the duplicated CAMV35S promoter

We designed two specific primers based on the nucleotide sequence of the duplicated CAMV35S promoter which drives the expression of the *NPTII* gene in the pCAMBIA2300 plasmid with sequences 35S.1: 5'-CCCCAAGCTTATGGTGGAGCACGACACT-3' and 35S.2: 5'-AAACGCAGAGAGAGAGATAGATTTGTAGAG-3'. 35S.1 and 35S.2 contain *HindIII* and *PstI* restriction sites, respectively, for cloning purpose. These primers were used to PCR-amplify the duplicated 35S promoter using pCAMBIA2300 plasmid DNA as template. PCR products were gel purified, cloned in pGEM-T easy vector and sequenced (double-strand) using ABI PRISM automated sequencer.

Construction of expression cassettes

Three expression cassettes were prepared: (1) for single expression of the *TVPI*, the *TVPI* gene, together with the 35S terminator, was cut from pCB302.2-*TVPI* plasmid (Brini et al. 2007) using *BamHI* and *KpnI* restriction enzymes and cloned into the multi-cloning site of pCAMBIA2300 using the same restriction sites. The resultant plasmid was named pCAMBIA-*TVPI*. The duplicated 35S promoter was cut from pGEM-Teasy vector using *HindIII/BamHI* restriction enzymes and cloned in front of the *TVPI* gene in pCAMBIA2300 using *HindIII/BamHI* restriction sites. The resultant plasmid was named herein pCAMBIA-35S-*TVPI*. (2) For single expression of the *TNHXS1* gene, three steps were used. First, the duplicated 35 S promoter was cut from pGEM-Teasy vector using *HindIII/PstI* restriction enzymes and cloned in the multi-cloning site of pCAMBIA2300 using the same restriction sites. The resultant plasmid was named herein pCAMBIA-35S. Second, the cDNA fragment of *TNHXS1* was cut from pGEM-Teasy vector using *PstI/XbaI* and cloned downstream of the CAMV35S promoter using the same restriction sites. Finally, the 35S terminator region was cut from pCAMBIA-*TVPI* (see above) using *XbaI/KpnI* restriction enzymes and cloned downstream of the *TNHXS1* in pCAMBIA-35S using the same restriction sites. The resultant plasmid was named herein pCAMBIA-

35S-*TNHXS1*, (3) for the bicistronic expression of *TVPI* and *TNHXS1*, the *TNHXS1* cDNA together with the 35S promoter were excised from pCAMBIA-35S-*TNHXS1* using *HindIII/XbaI* and the IRES was excised from pGEM-Teasy using *XbaI/BamHI* restriction enzymes. These two fragments were gel purified and used in a triple ligation reaction with pCAMBIA-*TVPI*, double-digested with *HindIII/BamHI* restriction enzymes, as recipient plasmid. The resultant construct was named TP3 (*TNHXS1*-IRES-*TVPI*).

Agrobacterium strain and tobacco transformation

Agrobacterium tumefaciens strain LBA4404 was transformed with the above-mentioned constructs using the chemical method and used for tobacco (*Nicotiana tabacum* var Xanthi) transformation by the leaf disc method (Horsch et al. 1985).

RT-PCR analysis

Total RNA was isolated from approximately 200 mg leaf tissue of transgenic tobacco lines and non-transformed control plants using the Trizol method (InVitrogen) and following the manufacturer instructions. RNA was treated with RNase-free DNase to remove any contaminating DNA. Reverse transcription reactions were performed for 1 h at 37°C using MML-Reverse transcriptase (InVitrogen) and oligo-dT. First strand cDNA was used as template for PCR amplifications using the following sets of specific primers: (1) *TNHXS1*Fw 5'-CTTCACCAGCACCGTTCT-3' and *TNHXS1*Rev 5'-TGCTCCTCTCATGAGACC-3', for *TNHXS1*, (2) *TVP*-Fw 5'-GTTCTTTACATCACCATC-3' and *TVP*-Rev 5'-CTCAACCATCTTGAGAGC-3' for *TVPI*. To verify that a full-length bicistronic RNA was produced in double transgenic plants, the following primers were used: TN.1: 5' CATTAGCTGCAGATGGGGCTCGATTGTTGGA and TVP.2: 5'-CTAATGTCTAGACTTCTAGATGTACTTGAA-3'. *TNHXS1*-Fw 5'-CTTCACCAGCACCGTTCT-3' and SP.2 (see above) and SP.1 and *TVP*.2 5'-CTAATGTCTAGACTTCTAGATGTACTTGAA-3'. The actin gene fragment, used as internal control was amplified with the primers: TAct1F 5'-GCCTCTTAACCCAAAGGCTAAT-3' and TAct1R 5'-TACCCCTCATAAATTGGGACAG-3'. Control amplifications in the absence of the reverse transcriptase were also performed to rule out any

1 amplification caused by the presence of contaminating DNA.

2 3 Isolation of tonoplast vesicles and assay of Na^+/H^+ exchange and H^+ -PPase activities

4
5 Tonoplast vesicles were isolated from tobacco roots as described by Barkla et al. (1999). The
6 measurement of cation/proton exchange was performed by monitoring the formation and dissipation of
7 pH gradients using ACMA (9-amino-6-chloro-2-methoxy-acridine) as indicator. Purified tonoplast
8 vesicles (50 μg of protein) were added to a buffer containing 250 mM mannitol, 10 mM BTP-MES (pH
9 8.0), 100 mM tetramethyl ammonium chloride, 3 mM MgSO_4 and 1 μM ACMA (1 ml final volume).
10 Formation of acid-inside pH gradients were started with the addition of 1.5 mM ATP-BTP (pH 8.0).
11 When fluorescence stabilized, the initial rate of dissipation was measured after the addition of NaCl or
12 KCl salts. The measurement of H^+ -PPase was performed following Maeshima and Yoshida (1989). A
13 similar amount of vesicles were suspended in a reaction medium consisting in 25 mM Hepes/BTP (pH
14 7.2), 0.25 M sorbitol, 1 mM MgSO_4 , 50 mM KCl, 1 μM ACMA and the reaction started with the
15 addition of 1 mM Na-pyrophosphate. Fluorescence was recorded with a Hitachi Fluorescence
16 Spectrophotometer (FL-2500) in a thermostated cell (26°C) at excitation and emission wavelengths of
17 415 and 485 nm respectively (slit-width, 10 nm). The reaction mixture was stirred and maintained at
18 26°C throughout the transport assays. Exchange rates are expressed as fluorescence recovery relative to
19 fluorescence values prior to ATP (or Na-pyrophosphate) addition ($\Delta F/F_{\text{max}}$), per minute and milligram
20 of protein.

21 22 Growth conditions and stress treatments

23
24 To compare germination rates under salt stress, seeds of wild type and T_3 transgenic tobacco plants
25 were sterilized using 70 % ethanol for 5 min, 50 % bleach for 10 min and washed five times with
26 sterile water. Sterile seeds (100 seeds) were sown on phyta-gel plates containing MS medium
27 (Murashige and Skoog, 1962) supplemented or not with 250 mM NaCl. The plates were incubated in
28 growth room at 23 °C and 16/8 hr photoperiod. The germination was measured after 2 weeks
29 incubation.

30 For evaluation of salinity tolerance at seedling stage, seeds of the wild type were germinated

on MS medium and those of the T₃ transgenic lines on MS supplemented with 250 mg/L kanamycin. Kanamycin-tolerant seedlings were transferred to MS medium supplemented with various concentrations of NaCl (0, 100 and 200 mM). Plates were held vertically. After 45 days of salt treatment, the seedlings were used to measure the parameters such as root length, fresh and dry weight, chlorophyll content and ion content.

To evaluate salinity tolerance under greenhouse conditions, one-month-old plants of transgenic lines and WT plants were transferred to pots containing a mixture of garden soil and sand (3:1) and placed in greenhouse. Salt stress was initiated two weeks later. NaCl concentrations were stepped up in 50 mM daily increments until the final concentration of 250 mM was achieved. Each line was irrigated twice a week with 250 mM NaCl solution. After 30 days of salt treatment, the plants were used to measure the various parameters such as plant weight and height, root length, catalase activity, electrolyte leakage of leaf cells, and relative water content (RWC).

Determination of relative water content (RWC)

Leaves were harvested and their fresh weight (FW) was determined immediately. They were floated on deionised water overnight at 4 ° C and their turgescence weight (TW) was recorded. Finally, leaves were dried at 70 ° C and their dry weight (DW) was recorded. Relative water content was calculated using the following formula: $RWC (\%) = (FW - DW / TW - DW) \times 100$

Determination of chlorophyll content

Chlorophyll was extracted as described in Arnon (1949) and content was calculated using formulas established by McKinney (1941).

Measurement of catalase activity

Plant material was homogenized in mortar on ice using pre-cooled (50 mM phosphate buffer (pH 7.0), 2 % PVP and 1 mM PMSF). The homogenate was centrifuged at 10 000 g for 20 min at 4 °C. Total soluble proteins (crude enzyme extract) were determined using Coomassie brilliant G250 according to

Bradford (1996) and BSA was used for standard curve. Catalase activity was measured according to Lück (1965). Briefly, three milliliter reaction mixtures consisting of 50 mM phosphate buffer (pH 7.0), 30 mM H₂O₂ and 100 µl of crude enzyme extract were used. The activity was determined by measuring the rate of disappearance of H₂O₂ for 2 min at 240 nm and quantified by its molar extinction coefficient and expressed as CAT units min⁻¹mg⁻¹ protein.

Determination of fresh and dry weights and ion contents

Fresh weights were determined immediately at the end of the salt treatment. Plant material was kept in an oven at temperature 70 °C until constant mass was reached than dry weights were determined. For ion content determination, plants were washed thoroughly in distilled water and then were separated in roots and shoots and oven dried as described above. Dry material was mineralised using 0.5 N HNO₃. After centrifugation to remove debris, the supernatants were analysed by atomic absorption spectrometry.

Determination of speed of Na⁺ uptake by leaf discs of transgenic tobacco lines

The Na⁺ absorption was done essentially as described by Gao et al. (2006). Briefly, healthy and fully expanded leaves were used to cut 30 leaf discs of 1 cm of diameter from wild type and transgenic plants. They were floated for 2 hr on distilled water and transferred thereafter to 300 mM NaCl solution and placed under continuous light at 23 °C. Six leaf discs from each line were retrieved at 2 h intervals, rinsed twice with distilled water, transferred to a solution of 100 mM KCl and 200 mM mannitol and floated for 20 min to remove NaCl from mesophyll cells. Thereafter, leaf discs were rinsed again twice with water, dried and used for determination of sodium content by atomic absorption as described above. The speed of absorption of Na⁺: Na⁺ uptake speed (ppm h⁻¹ per disc) = (T_{N+2} Na⁺ content – T_N Na⁺ content)/2 where T_N Na⁺ content presents Na⁺ content per disc at different time points.

Leaf disc senescence assay

Leaf discs of 1 cm of diameter were excised from healthy and fully expanded tobacco leaves of WT

1 and various transgenic lines of similar age. Leaf discs were floated in 10 mL of NaCl solution (300 and
2 500 mM, 3 and 5 days) or sterile distilled water.

3

4 Determination of leaf cell membrane damage

5

6 The leaf cell membrane damage was measured as electrolyte leakage from leaf cells using the
7 following formula: electrolyte leakage = $(C1/C2) \times 100$, where C1 and C2 represent the conductivity
8 measurement of non-boiled and boiled leaves for 1 hr, respectively.

9

10 Statistical analysis

11

12 Data were analysed using one-way ANOVA implemented in the SPSS software 13. Treatment mean
13 separations were performed using Duncan test.

14

Results

Cloning of the *TNHXS1* and its functional analysis in yeast

A pair of primers TN.1 and TN.2, was designed to amplify *TNHXS1* using a wheat (*Triticum aestivum*) root tissue cDNA library. A DNA fragment of 1,650 bp was obtained and sequenced. The result indicated that the cDNA encoded a polypeptide of 549 amino acid residues that contained 12 putative transmembrane domains and the binding site of amiloride, the signature of Na⁺/H⁺ antiporter family (data not shown). To gain information about the function of *TNHXS1*, the full-length cDNA was cloned in pYES2 vector and introduced into the yeast strain AXT3 in which all major endogenous sodium transporters essential for salt tolerance are disrupted. Cells of AXT3 were grown on YPG supplemented with NaCl (100 mM), KCl (1 M) and hygromycin (20µg/ml). As shown in Fig. 1a, AXT3 harbouring the pYES2 empty vector was more sensitive to NaCl, KCl and hygromycin than AXT3 transformed with pYES2-*TNHXS1*. In liquid medium containing 100 mM NaCl and 1 M KCl, *TNHXS1*-expressing yeast grew better than the control yeast strain (Fig. 1b). The total ion contents of AXT3 yeast cells harbouring pYES2 empty vector and pYES2-*TNHXS1* were determined. In the presence of 100 mM NaCl or 1M KCl in growth medium, the yeast strain harbouring the *TNHXS1* was able to accumulate more Na⁺ (Fig.1c, e) and K⁺ (Fig.1d, f) than that of the yeast harbouring pYES2 empty vector. These results indicate that *TNHXS1* is a functional antiporter capable of sequestering both Na⁺ and K⁺ in the vacuole of yeast and to improve salt tolerance.

Generation and molecular characterisation of transgenic tobacco plants

Transformation vectors containing *TNHXS1*, *TVPI* or *TNHXS1* together with *TVPI* were constructed. The T-DNA region of the various constructs is schematically presented in Fig. 2a. To co-ordinate the expression of *TNHXS1* and *TVPI* genes, we used an IRES element from plant origin, the *Nicotiana tabacum* 5'UTR of the heat-shock factor1 (*NtHSF-1*) mRNA. The activity of this IRES element in plants was previously demonstrated (Dorokhov et al. 2002). We designed a pair of primers SP.1 and SP.2 based on the nucleotide sequence of *NtHSF-1* to PCR-amplify the tobacco IRES using total genomic DNA. A single band of the expected size (453 bp) was successfully amplified, cloned and

sequenced (data not shown). NCBI blast showed that it has 99 % identity with the *Nicotiana tabacum* 5'UTR of the heat-shock factor1 (*NtHSF-1*) mRNA. This IRES was used as intercistronic spacer between the *TNHXS1* and the *TVPI* genes (Fig. 2a). Prior to transfer into *Agrobacterium* cells, the bicistronic construct was verified by restriction digestions. The junctions between the IRES and the C-terminal region of *TNHXS1* protein as well as between IRES and the N-terminal region of the *TVPI* protein were verified by sequencing (data not shown). Several T₀ kanamycin-resistant plants were obtained from transformations with the three gene constructs. Here we used the T₃ generation of these plants. Before use, the T₃ plants were first characterized using PCR amplification on total genomic DNA and the positive lines (data not shown) were characterised further using reverse transcriptase polymerase chain reaction (RT-PCR). For monotransgenic plants, specific primers corresponding to *TNHXS1* or *TVPI* genes were used. As shown in Fig. 2, specific bands of the expected sizes were obtained for *TNHXS1* transgenic plants (Fig. 2b, *TNHXS1cl13* and *TNHXS1cl17*) or *TVPI* transgenic plants (Fig.2c, *TVP1cl19* and *TVP1cl20*). These same primers were also used to amplify total RNA from the bicistronic plants and the expected specific bands of *TNHXS1* and *TVPI* were successfully amplified in TP3 plants (Fig 2d, *TNHXS1* and *TVPI*). However, nothing was obtained from the WT plants. To verify that a full-length of the bicistronic mRNA was produced in TP3 transgenic lines, we performed additional PCR amplifications using various sets of primers: (1) a forward primer specific to the N-terminal region of *TNHXS1*, including the ATG start codon and a reverse primer located at the end of *TVPI* gene, including the stop codon (TN.1/TV.P.2 see Fig 2a) that would amplify the full-length bicistronic RNA, (2) a second pair of primer (*TNHXS1*/IRES) that would amplify part of the *TNHXS1* and the full-length IRES, and (3) a third primer set (IRES/*TVPI*) that would amplify the full-length IRES along with the full-length *TVPI* gene (Fig. 2a). All of the three sets of primers successfully amplified fragments of the correct sizes (Fig. 2d). These results indicate that the different transgenes were correctly integrated into the tobacco genome and that the corresponding mRNA was stably produced through generations T₀-T₃. To facilitate an in-depth study of mono- and double-transgenic plants, we selected two transgenic lines harbouring the *TNHXS1* gene (lines *TNHXScl3* and *TNHXScl17*), the *TVPI* gene (*TVP1cl19* and *TVP1cl 20*) and both genes (*TP3cl38* and *TP3cl44*).

Antiport activity in tonoplast vesicles

To demonstrate enhanced activity of the H⁺-PPiase (TVP1) and Na⁺/H⁺ antiporter (TNHXS1) proteins in the transgenic lines, vacuolar membranes were purified from tobacco roots and subjected to ion transport analysis by monitoring the formation and dissipation of pH gradients using the fluorescent probe ACMA. Proton pumping was measured in the presence of either pyrophosphate or ATP as substrates for the H⁺-PPiase and V-ATPase, respectively. To measure Na⁺/H⁺ and K⁺/H⁺ antiport activity, vesicles were energized with ATP and the endogenous V-ATPase to obtain similar initial proton gradient regardless of the presence of ectopically expressed TPV1 in the tonoplast vesicles of lines TVP1cl19 and TP3cl38.

The TP3 transgenic lines showed significantly greater activity of the H⁺-PPiase and vacuolar H⁺-ATPase than the WT line (Fig. 3a, b). The single transgenic lines showed higher H⁺-PPiase (TVP1) and Na⁺/H⁺ antiport (TNHXS1) than the control line (WT). Interestingly, the *TVPI* line also showed increased antiport activity in comparison with the WT (Fig. 3c) and the H⁺-PPiase activity was much higher in the double-transgene line TP3 than in the transgenic line expressing TVP1 alone (Fig. 3a), suggesting that these two proteins interacted functionally in a positive feed-back. Biochemical characterization of NHX proteins has shown that, depending on the specific isoform, these proteins do not discriminate between Na⁺ and K⁺ in exchange for H⁺, or they have a preference for K⁺ over Na⁺ (Jiang et al., 2010; Rodriguez-Rosales et al., 2009). While some degree for Na⁺ preference might be inferred in vesicles from WT plants, both Na⁺/H⁺ and K⁺/H⁺ exchange activity were enhanced in the TP3 transgenic line, indicating lack of Na⁺-K⁺ discrimination or even a K⁺ preference in *TNHXS1* (Figure 3d).

TP3 transgenic plants have better growth and development under *in vitro* conditions

To evaluate the impact of the co-expression of *TNHXS1* and *TVPI* genes on salinity tolerance, we first conducted an *in vitro* experiment to compare seed germination, total plant biomass, dry weight, root length and chlorophyll content in TP3 and monotransgenic plants. As illustrated in Fig. 4a-d, in MS medium and when subjected to 100 mM NaCl treatment, root lengths of TP3 and *TVPI* transgenic

1 plants were similar, but were significantly higher than that of the wild type and *TNHXSI* transgenic
2 plants. The TP3 plants, however, exhibited significantly longer roots than the monotransgenics,
3 including *TVPI* transgenics, when subjected to 200 mM NaCl treatment (Fig. 4d).

4 In the absence of salt stress, no significant difference was detected between total fresh weights (roots
5 and shoots) of TP3 and *TVPI* seedlings (Fig. 4e). However, the total fresh weights of TP3 and *TVPI*
6 plants were significantly greater than that of the wild type and *TNHXSI* transgenic seedlings. At 100
7 mM NaCl, no significant difference in fresh weight was detected between TP3 and monotransgenic
8 plants. In contrast, at 200 mM, TP3 seedlings exhibited significantly higher fresh weight than
9 monotransgenic seedlings. Concerning dry weight, no significant difference was detected between the
10 three transgenic lines when plants are grown on MS medium. In contrast, when subjected to 100 mM
11 and 200 mM of NaCl treatment, the TP3 plants exhibited significantly greater dry weight than the
12 monotransgenic plants. The ectopic expression of *TNHXSI* and *TVPI* genes appeared to enhance
13 growth and development of transgenic plants even in the absence of salt stress (Fig. 4a). Thus, to
14 demonstrate that the difference in performance between WT and various transgenic lines was also due
15 to an enhanced salinity tolerance, we calculated the percentage reduction compared with the respective
16 unstressed control for the different growth parameters (fresh weight, dry weight and root length).
17 Results showed that the percentage reduction under salinity stress was in the following order: WT
18 plants > *TNHXSI* plants > *TVPI* plants > TP3 plants. The effect of the co-expression of *TNHXSI* and
19 *TVPI* genes on seed germination was also evaluated. The different transgenic lines and the wild type
20 had similar seed germination under non stress conditions. In contrast, when subjected to 250 mM, seed
21 germination was reduced by 75 %, 6%, 8 % and 5% in WT, *TNHXSI*, *TVPI* and TP3 transgenic lines,
22 respectively.

23
24 The effect of salt treatment on Na⁺ and K⁺ accumulation in TP3 and monotransgenic plants grown *in*
25 *vitro*

26
27 The content of Na⁺ and K⁺ were determined in leaves and roots of plants from various transgenic lines
28 and WT plants grown under different NaCl concentrations (0, 100 and 200 mM). As shown in Fig. 5,
29 the Na⁺ contents showed little difference between the various transgenic lines and the WT plants under
30 non salt-stressed conditions. Both in leaves and roots, the Na⁺ concentration increased with increasing

NaCl concentration from 0 to 200 mM. However, Na⁺ content of leaves increased to higher level in the WT plants than that of the different transgenic lines including the TP3 plants (Fig. 5a). The latter exhibited the lowest Na⁺ content compared with the WT, the *TNHXS1* and the *TVPI* transgenic plants. In the absence of salt treatment, no significant difference was detected between K⁺ concentrations in leaves of the WT and *TVPI* transgenic plants, but *TNHXS1* and TP3 plants accumulated significantly higher K⁺. With increasing the NaCl concentration, K⁺ content in leaves of the WT plants decreased significantly, increased slightly in the case of the *TNHXS1* and *TVPI* transgenic plants, and doubled in the TP3 transgenic plants (Fig. 5c). In roots, we observed a contrasting behaviour of Na⁺ accumulation compared with leaves. As shown in Fig. 5d, all the transgenic lines accumulated more Na⁺ in their roots compared with the WT plants under salt stress. Interestingly, Na⁺ concentration in roots of the TP3 transgenic plants was significantly higher than that of the *TNHXS1* and the *TVPI* transgenic and the WT plants. When subjected to 100 and 200 mM NaCl treatment, the WT plants exhibited a significant decrease in K⁺ content of their roots with increasing NaCl concentration, the *TNHXS1* and the *TVPI* transgenic plants showed slight decrease, whereas the TP3 transgenic plants exhibited the largest increase of K⁺ content (Fig. 5d). For both NaCl concentrations (100 and 200 mM), the TP3 transgenic line showed the highest accumulation of K⁺ in its roots compared with the *TNHXS1* and the *TVPI* transgenic plants (Fig. 5d). Interestingly, the K⁺/Na⁺ ratio in the roots and shoots of the transgenic plants was higher than that in the WT plants and was in the following order: TP3 (bicistronic) > *TNHXS1* > *TVPI* > WT, suggesting that the over-expression of *TNHXS1* antiporter alone or in combination with *TVPI* may be effective in the accumulation of K⁺ in vacuoles.

Leaf discs of TP3 transgenic lines showed faster Na⁺ uptake than monotransgenic lines

Accumulation of Na⁺ in shoot is controlled by multiples mechanisms. These include exclusion of Na⁺ from uptake by roots, vacuolar compartmentation in root tissue and control of Na⁺ loading into the xylem. To eliminate the contribution of these mechanisms, we measured the speed of Na⁺ uptake using leaf discs derived from transgenic plants grown under normal conditions. Leaf discs from the TP3 transgenic plants showed faster uptake of Na⁺ than that of the *TNHXS1* and *TVPI* transgenic plants. The calculated speed of Na⁺ uptake by the leaf discs was 0.70, 1.70 and 2.26 (ppm.h⁻¹ per disc) for the *TVPI*, *TNHXS1* and the TP3 transgenic plants, respectively.

1 TP3 plants exhibited better performance under greenhouse-based salt stress

2
3 To evaluate further the salinity tolerance conferred by the dicistronic construct, we conducted a
4 greenhouse experiment and scored different growth parameters such as plant fresh weight, plant
5 weight, number of leaves per plant, root lengths and seed production. Under non-stress conditions, the
6 different transgenic lines showed normal phenotypes and their growth parameters were even superior to
7 those of the WT plants (Fig. 6 and Table 1). When subjected to 250 mM salt stress, the TP3 and the
8 *TVPI* transgenic plants exhibited similar fresh weight production, plant heights, and seed production.
9 However, TP3 and *TVPI* transgenic lines significantly outperformed the WT and the *TNHXSI*
10 transgenic plants with regard to these parameters (Table 1). The TP3 plants were also found to produce
11 significantly more leaves than the monotransgenic lines and the WT plants (Table 1). It is worth
12 mentioning here that under both conditions (salt and non-salt stress), the TP3 plants showed better
13 developed root systems (Fig. 6 and Table 1). To confirm further the improved salt stress tolerance of
14 the transgenic lines, we calculated the percentage reduction compared with the respective unstressed
15 control for the various parameters. At 250 mM NaCl, a reduction of 80 % in plant fresh weight was
16 registered in WT plants, whereas in *TNHXSI*, *TVPI* and TP3 transgenic lines, the plant fresh weight
17 percentage reduction was 61 %, 61 % and 54 %, respectively. The percentage reduction in plant height
18 was 76 % for the WT, whereas in *TNHXSI*, *TVPI* and TP3 transgenic lines, the plant height
19 percentage reduction was 63 %, 52 % and 32 %, respectively. The number of leaves per plant followed
20 the same trend with the highest percentage reduction registered in WT plants and the lowest in TP3
21 transgenic plants.

22
23 TP3 transgenic plants retained more chlorophyll under salt stress

24
25 Non-stressed plants grown in the greenhouse were analysed for tolerance to NaCl stress by leaf disc
26 senescence assay. Incubation of leaf discs of the WT, the TP3, the *TNHXSI* and the *TVPI* transgenic
27 plants on 300, 500 mM NaCl and water for 72 h showed an early bleaching of the WT leaf discs
28 compared with transgenic plants as shown in Fig. 7a. At both concentrations of salt, the decrease in
29 chlorophyll content in the transgenic plants was less pronounced than in the WT plants.

30 We also measured the total chlorophyll content of the various transgenic seedlings grown *in vitro* and

found that without salt treatment, TP3 transgenic lines exhibited slightly higher chlorophyll contents compared with the *TNHXS1*, *TVPI* and the WT plants (Fig. 7b). Salt treatment quickly decreased total chlorophyll content of the WT, *TNHXS1* and *TVPI* transgenic plants. However, the extent of this decline was much less in the TP3 transgenic plants. For example, at 100 mM NaCl treatment, chlorophyll content in TP3 (line 44) was similar to that recorded in the absence of salt treatment (Fig. 7b). Although TP3 transgenic plants retained less chlorophyll at 200 mM salt treatment than at 100 mM, their chlorophyll content was still higher than that of the other transgenic lines and the WT (Fig. 7b). Taken together, these results indicated that the photosynthetic capacities of the TP3 transgenic plants remained higher during salt treatment than those of the transgenic plants over-expressing *TNHXS1* or *TVPI* genes individually.

TP3 transgenic plants maintained high catalase activity under salt stress

Salt stress induces cellular accumulation of reactive oxygen species ROS which can damage membrane lipids, proteins and nucleic acids. Catalase enzymes that can remove the ROS produced by the salt stress play an important role in tolerance. Thus, we measured CAT activity in the leaves of the WT and the various transgenic lines subjected to salt stress. The results indicated that in the absence of salt stress, the *TNHXS1* and *TVPI* transgenic plants exhibited over 3-fold more CAT activity in leaf crude extracts compared with the WT plants. The TP3 transgenic lines had over 1.5-fold more CAT activity than the *TNHXS1* and the *TVPI* transgenic lines (Fig. 8a). When subjected to 250 mM NaCl salt treatment, CAT activity decreased in both the WT and the different transgenic lines, but CAT activity remained highest in the TP3 transgenic lines (Fig. 8a). Compared with control conditions (absence of salt), the decrease in CAT activity was 84% in the WT plants, 44% in the *TNHXS1*, 33% in the *TVPI* transgenic plants, and only 25% in the TP3 transgenic plants.

TP3 transgenic plants exhibited minimal membrane damage under salt stress

To study the stability of membrane in transgenic plants under salt stress, we determined the electrolyte leakage (%) which represents the degree of cell membrane damage under stress. The results indicated that membrane permeability increased when plants are subjected to 250 mM salt stress, but it showed a

1 faster rise in the WT plants than that in the transgenic lines. The damage to the TP3 transgenic lines
2 was less than to the single transgenic lines, *TNHXS1* and *TVPI*, which had significantly less damage
3 than the WT (Fig. 8b). In fact, after one month of salt treatment, the electrolyte leakage was 83 % for
4 the WT. However, it was 70 % in the *TNHXS1* transgenic lines, 60 % in the *TVPI* and only 45 % in the
5 TP3 lines.

6
7 TP3 plants retained more water than mono-transgenic lines under salt stress

8
9 In order to assess the water status of both the TP3 and the mono-transgenic plants, we measured the
10 relative water content (RWC) of plants. As shown in Fig. 8c, no significant difference in RWC was
11 observed between the WT and the various transgenic lines before application of salt stress. When
12 exposed to salt treatment of 250 mM NaCl, significant differences in RWC were detected between the
13 WT and the different transgenic lines, indicating greater water retention capacity in the transgenic
14 plants than in the WT. Under salt treatment, the RWC of the TP3 and the mono-transgenic plants
15 declined, but this decline was more important in the *TNHXS1* and *TVPI* transgenic plants compared
16 with the bicistronic TP3 lines.

17 18 **Discussion**

19 Salinity tolerance is a complex, multi-genic trait which involves many physiological and biochemical
20 mechanisms. Fortunately, a large number of potentially important genes which confer salinity tolerance
21 have been identified using forward and reverse genetics, yeast complementation and transcriptomics
22 approaches. Although the over-expression of a single salt-responsive gene can contribute to the
23 improvement of salinity tolerance to some extent, the level of salt resistance is still below the level
24 needed for practical agriculture production. In addition, it has been observed that in many cases, the
25 overall stress tolerance imparted by over-expression of a single gene may not sustain itself under field
26 conditions (Singla-Parek et al. 2007). Therefore, more integrated approaches are urgently needed. One
27 approach consists of simultaneous up-regulation of extruding mechanisms through over-expression of
28 systems such as vacuolar pumps, Na⁺ exchangers (NHXs and SOS1) and loss-of-function mutations in
29 uptake pathways such as non-selective ion channels and HKTs. This is feasible through the use of
30 polycistronic expression vectors and gene stacking methodology. In the present study, we used a

dicistronic vector for the co-expression of two wheat genes, *TNHXS1* and *TVPI*, in transgenic tobacco plants. We demonstrated that simultaneous over-expression of these two tonoplast proteins conferred better salt tolerance in transgenic tobacco plants than the over-expression of single genes.

The main objective of our cloning strategy was to physically link the *TNHXS1* and the *TVPI* genes and positioning them contiguously on DNA that will transfer as a single entity into tobacco genome. We also aimed to express those genes from a single 35S promoter to avoid the repetitious promoter use that leads, in some cases, to negative impact on transgene stability and expression (Peremarti et al. 2010). This strategy needs an important element known as internal ribosome entry sites (IRESs) which are usually viral sequences that can directly recruit ribosomes to internal positions within mRNAs and initiate translation in a cap-independent manner. Various IRES elements of different origins were described in the literature (Halpin, 2005). We have chosen and used an IRES element which was previously shown to be functionally active in tobacco protoplasts and human Hela cells transgenic for dicistronic constructs involving the two reporter genes *GFP* and *GUS* (Dorokhov et al. 2002). Thus, we expected the IRES to be effective in co-ordinating the expression of the *TNHXS1* and the *TVPI* genes in transgenic tobacco plants. Indeed, molecular analysis of the transgenic tobacco plants demonstrated the integration of the transgenes and the production of the full-length bicistronic mRNA which contains the first cistron *TNHXS1*, the IRES element essential for the internal translation of the second cistron and the second cistron *TVPI*. More important, the H⁺-pyrophosphatase activity measured in the double transgenic plants was twice that of the WT and the single *TVPI* transformants, which demonstrates that the IRES element used in the present study was very efficient. In a previous study, dicistronic analysis of IRES activity of the 5'UTR of *NtHSF1* mRNA (used in this study) in tobacco protoplasts revealed activity comparable to those mediated by IRES of crucifer-infecting tobamovirus (CrTMV) (Dorokhov et al. 2002). CrTMV promotes the translation of a second cistron at 21-31 % of the levels of the first cistron when a bicistronic construct is expressed in transgenic tobacco plants (Dorokhov et al. 2002). Recently, Ha et al. (2010) have also demonstrated that the IRES of CrTMV can coordinate the expression of two biosynthetic genes in transgenic rice.

The prevailing view is that the compartmentation of Na⁺ into the plant vacuoles represents an efficient mechanism to avoid toxic effects of Na⁺ ion in the cytosol (Apse and Blumwald, 2007). The transport of Na⁺ into vacuoles is mediated by cation/H⁺ antiporters that are driven by electrical gradient of protons generated by the vacuolar H⁺ pumps. A large number of genes encoding NHX-like vacuolar

Na⁺/H⁺ antiporters have been isolated from diverse plants and successfully used to enhance salt tolerance in transgenic plants (reviewed in Jiang et al., 2010; Rodriguez-Rosales et al., 2009; Ashraf and Akram, 2009). However, the salt tolerance imparted by these antiporters varied with the source of antiporter and the plant species engineered. A recent study (Liu et al. 2010) reported the expression, in *Arabidopsis thaliana*, of five NHXs from different origins including one from yeast *ScNHX1*, two from dicotyledons (*Arabidopsis thaliana*, *AtNHX1* and *Gossypium hirsutum*, *GhNHX1*) and two from monocotyledons (*Triticum aestivum*, *TaNHX1* and *Suada salsa*, *SsNHX1*). It was found that the ectopic expression of these NHXs in *Arabidopsis thaliana* caused growth retardation in transgenic *Arabidopsis* compared with wild type plants under normal growth conditions. Under stress conditions, the various NHX proteins conferred higher salt tolerance to transgenic *Arabidopsis* plants compared to wild type plants, but the *TaNHX1* from wheat was less efficient than the other antiporters. Here, the ectopic expression of *TNHXS1* in tobacco did not cause any growth retardation in transgenic lines compared to wild type plants. It is worth mentioning that *TNHXS1* has 99 % identity to *TaNHX1* used in Liu et al. (2010). Transgenic *TNHXS1* plants exhibited better growth and physiological parameters than the wild type plants (Fig. 6 and Table 1) under both normal and salt stress conditions. Interestingly, under high salt conditions, *TNHXS1* transgenic plants were able to complete their life cycle and to produce viable seeds whereas WT plants died at the vegetative stage. In addition, *TNHXS1* was found to complement a salt-sensitive yeast mutant, and to be able to transport both Na⁺ and K⁺ into the vacuole of yeast strains, indicating that *TNHXS1* is a functional antiporter (Fig. 1). These results indicated that *TNHXS1* was a likely candidate gene for enhancing the salt tolerance of transgenic plants.

In the present study, it was observed that the various transgenic lines exhibited better growth than WT plants under normal conditions (Table 1). These transgenic lines expressed constitutively, under the control of the strong 35S promoter, an NHX-type antiporter or a vacuolar H⁺-pyrophosphatase, singly or in combination. These results are not surprising, since it was previously demonstrated that plant vacuolar H⁺-pyrophosphatase plays an important role in plant organogenesis through control of auxin transport and distribution (Li et al., 2005). In fact, over-expression of plant vacuolar H⁺-pyrophosphatase enhanced both shoot and root development in transgenic plants of tomato (Park et al., 2005; Khoudi et al., 2009), cotton (Asad et al., 2008) corn (Li et al., 2008), and bentgrass (Li et al., 2010). A robust root system facilitates water uptake when plants are subjected to abiotic stresses such as drought and salinity. Thus, the TP3 plants described in the present investigation would also be

1 drought tolerant. In addition, the protons pumped into the central vacuole by the tonoplast
2 pyrophosphatase are used for cation accumulation which drives the cell growth. On the other hand,
3 Bassil et al. (2011) demonstrated the role of NHXs in plant growth and development using reverse
4 genetics. It was shown that NHXs control cell expansion in vegetative tissues and male reproductive
5 organs and are required for normal flower development. In fact, *nhx1 nhx2* double mutant plants were
6 significantly smaller than wild type, and this was associated with a decrease in cell size rather than
7 defects in tissue organization. According to these authors, NHX-type transporters participate in cation
8 accumulation in the central vacuole leading to water uptake and thus to cell expansion and growth.

9 Although NHX proteins are generally regarded as Na^+/H^+ antiporters, recent evidence
10 indicates that they catalyze substantial K^+/H^+ exchange in plants (Leidi et al. 2010; Rodriguez-Rosales
11 et al. 2009). Interestingly, the Na^+ content in the leaves of the *TNHXS1* transgenic plants was
12 significantly lower than that of the wild type plants, whereas the K^+ content increased compared to wild
13 type plants under saline stress (Fig. 5a, c). Notably, the K^+ content in leaves of salt-stressed TP3 plants
14 more than doubled compared to these plants in the absence of NaCl. In contrast, roots of the *TNHXS1*
15 transgenic plants contained higher Na^+ content than roots of the wild type plants. Similar findings by
16 several authors (Liu et al. 2010; Xue et al. 2004; Zhao et al. 2007), reporting over-expression of NHX
17 proteins, were regarded as a consequence of higher Na^+ transport into root vacuoles of the transgenic
18 lines which led to a lower Na^+ level in the cytosol and intracellular fluid of roots for transport into the
19 shoot through the xylem transpiration stream. However in the light of recent reports (see below) it is
20 more likely that over-expression of *TNHXS1* led to a greater control of K^+ homeostasis that minimised
21 Na^+ uptake mechanisms in the first place. In the present study, we found that, under 200 mM salt
22 treatment, *TNHXS1* and *TP3* roots accumulated 2.2 and 2.7 times, respectively, more Na^+ than wild
23 type plants, whereas they accumulated 3.1 and 4.5 times, respectively, more K^+ than wild type plants
24 (Fig. 5). Again, TP3 lines accumulated more K^+ in their roots in the presence of NaCl than in control
25 conditions, opposite to what happened to wild type plants (Fig. 5d). Our results on Na^+ accumulation
26 are in disagreement with the most recent study, Liu et al. (2010), who reported a higher accumulation
27 of Na^+ in the leaves of transgenic plants, including transgenics harbouring *TaNHX1*, compared to WT
28 plants under 200 mM salt treatment. However, our results are in line with those reported by Islam et al.
29 (2010) who demonstrated that the over-expression of *Pennisetum glaucum* vacuolar antiporter gene
30 (*PgNHX1*) in rice led to significantly higher leaf content of K^+ and less Na^+ in transgenic plants as

1 compared to wild type plants. Similar results were also reported by Zhao et al. (2007) and Wei et al.
2 (2010). The over-expression of *HvNHX2* from barley in transgenic potato (Bayat et al. 2010) affected
3 ion accumulation in a similar way to that of the over-expression of *TNHXS1* in transgenic tobacco (this
4 study). Interestingly, sequence analysis revealed 99 % similarity between *HvNHX2* and *TNHXS1*.

5 Our results for *TVPI* transgenic plants are also in disagreement with previous studies (Gao et
6 al. 2006, Guo et al. 2006; Li et al. 2010) dealing with ectopic expression of V-PPiase proton pumps. In
7 fact, all these authors reported an enhanced Na^+ accumulation in the leaves of transgenic plants
8 compared to wild type plants, following salt treatment. In our hands, the analysis of ion contents
9 indicated that under stress treatments, Na^+ accumulation in wild type plants was significantly higher
10 than that in *TVPI* transgenic plants (Fig. 5a). Our results are in good agreement with Liu et al. (2010)
11 who reported less accumulation of Na^+ in the leaves of transgenic rice plants over-expressing rice
12 pyrophosphatase gene *OsVPI* singly or in combination with sodium antiporter gene *OsNHX1*.

13 Ion transport analyses with tonoplast vesicles isolated from transgenic lines confirmed that
14 single-transgenic lines TVP1cl19 and TNHXS1cl7 had greater H^+ -PPiase and Na^+/H^+ antiport activity,
15 respectively, than the WT. Interestingly, the double-transgene line TP3cl38 co-expressing both *TNHXS1*
16 and *TVPI* showed even greater H^+ -PPiase activity (Fig. 3). This enhancement did not appear to be
17 caused by different expression levels of the *TVPI* transgene among lines based on RT-PCR analyses
18 (Figure 2). Moreover, the single-transgene line TVP1cl19 also showed increased Na^+/H^+ antiport
19 activity relative to non-transformed WT plants (Fig. 3c) and the co-expression of *TVPI* and *TNHXS1*
20 induced the H^+ pumping activity of the endogenous V-ATPase (Fig. 3b). Thus, it appeared that
21 ectopically expressed *TVPI* and *TNHXS1* interacted in a synergistic manner to potentiate both H^+
22 pumps, the H^+ -PPiase and the V-ATPase. Activation of the V-ATPase could further contribute to the
23 enhanced stress tolerance of line TP3cl38. Indeed, many studies have demonstrated that the
24 enhancement of V-ATPase activity contributes to the improvement of salt stress tolerance in plants
25 (Kluge et al. 2003; Padmanaban et al. 2004; Guo et al. 2006; Batelli et al. 2007; Wang et al. 2011;
26 Zhou et al. 2011; Hu et al. 2012). Thus, transgenic TP3 plants, which over-expressed simultaneously
27 *TNHXS1* and *TVPI* genes, exhibited the best salinity tolerance compared to control plants when
28 challenged with salt treatment *in vitro* or under greenhouse conditions.

29 The Na^+/K^+ selectivity of the *TNHXS1* is not yet known. Comparison of Na^+/H^+ and K^+/H^+
30 exchange activity in WT and TP3 plants showed that whereas the tonoplast vesicles from WT had

greater Na^+/H^+ exchange than K^+/H^+ exchange, both activities were commensurately enhanced in the *TNHXS1* expressing plants (Fig. 3d), suggesting that this exchanger might have a preference for K^+ over Na^+ . When exposed to salt treatment (200 mM), elemental analysis revealed that TP3 plants accumulated 20 and 4.5 times more K^+ in their leaves and roots, respectively, compared to wild type plants (Fig. 5c, d). In addition, TP3 transgenic plants accumulated less Na^+ in their leaves but only 2.7 times more Na^+ in their roots when compared to wild type plants. The K^+ accumulation in the leaves and roots was enhanced in TP3 transgenic plants following the increase in salt concentration in the medium whereas it was decreased in the case of the wild type plants (Fig. 5c, d). The unusual accumulation of K^+ in the leaves of TP3 plants, as NaCl concentration increased in the culture medium, could be due to the activity of *TNHXS1* alone or because additional mechanisms involved in K^+ homeostasis were activated following the ectopic expression of *TNHXS1*. It has been established that *AtNHX1* influenced the expression of several genes including ion transporters (Sottosanto et al. 2004). Significantly higher K^+/Na^+ ratio in transgenic tobacco leaves is a strong indication of salt tolerance. Maintenance of higher K^+/Na^+ ratio in the transgenic *TNHXS1* and TP3 lines under salt stress can be attributed to improved K^+ homeostasis caused by over-expression of vacuolar antiporter (Gao et al. 2007).

The results obtained in this study raise the question of whether the *TNHXS1* is a Na^+/H^+ antiporter and whether it is playing any significant role in salinity tolerance through vacuolar sequestration of Na^+ . Clearly, our results indicate that the enhanced salt tolerance exhibited by *TNHXS1* and TP3 transgenic plants is more related to a stronger control of K^+ homeostasis than to increased vacuolar Na^+ sequestration. In other words, *TNHXS1* facilitates K^+ accumulation which in turn leads to salinity tolerance. This is very plausible given the fact that the role of NHX proteins in salinity tolerance through Na^+ vacuolar sequestration was revisited and an updated model was proposed (Jiang et al. 2010). According to this model, NHX proteins fulfil a protective function to minimize salt-related stress mainly through the vacuolar compartmentalization of K^+ and, in some cases of Na^+ as well, thereby preventing toxic Na^+/K^+ ratio in the cytosol while acquiring solutes for osmotic balance (Jiang et al. 2010). Many lines of evidence are in support of this model. First, the founding member of NHX-like antiporters, *AtNHX1* from Arabidopsis, was found to mediate Na^+/H^+ and K^+/H^+ exchange in tonoplast vesicles from transgenic tomato (Zhang and Blumwald, 2001), in artificial proteoliposomes (Venema et al. 2002) and in vacuoles of yeast mutant lacking the endogenous Na^+/H^+ and K^+/H^+

activities at the tonoplast (Hernandez et al. 2009). Second, in an experiment aiming at the determination of cation dependant H^+ exchange using lipid vesicles system, the *AtNHX1*, which is localised in the tonoplast, showed equal transport rates for Na^+/H^+ and K^+/H^+ exchange whereas the tomato, *LeNHX2*, which is targeted to the prevacuolar compartment, showed a strong preference for K^+/H^+ exchange over Na^+/H^+ (Jiang et al. 2010). Third, mutants of *AtNHX1* with improved K^+-Na^+ selectivity conferred halotolerance to yeast cells presumably by averting sodic poisoning of the endomembrane network and preserving endosome/prevacuolar functions (Hernandez et al. 2009). In tomato, over-expression of NHX proteins imparted salt tolerance through a mechanism involving K^+ compartmentation (Rodriguez-Rosales et al. 2009; Leidi et al. 2010). On the other hand, it was demonstrated that NHX proteins mediate K^+/H^+ exchange for turgor and pH control especially in petal expansion and flower colour development (Yoshida et al. 2009). The results obtained herein are therefore more easily explained based on the model proposed by Jiang et al. (2010) and indicate that *TNHXSI* is mainly a K^+-H^+ antiporter, although it may also mediate some Na^+ sequestration activity.

The co-expression of the *TNHXSI* and *TVPI* genes conferred higher tolerance to salt stress than single expression of the *TNHXSI* or *TVPI* genes. Our results are, therefore, in agreement with previous studies reporting the co-expression of vacuolar Na^+/H^+ and pyrophosphatase genes (Zhao et al. 2006; Liu et al. 2010). Interestingly, our results seem to indicate that ectopically expressed *TVPI* and *TNHXSI* interacted in a synergistic manner to potentiate both H^+ pumps. This would result in an increased electrochemical gradient to promote the exchange of Na^+-K^+/H^+ activity leading to the sequestration of K^+ by *TNHXSI*. In a recent study on the salinity tolerance of *Arabidopsis* ecotypes, Jha et al. (2010) have shown that the up-regulation of *AtNHX1* and *AtVPI* was positively correlated in response to salt stress in both shoots and roots. In this study, the TP3 plants exhibited the least Na^+ content in leaves, the highest Na^+ content in roots, and the highest level of K^+ in their leaves and roots when compared to single *TNHXSI* or *TVPI* transgenic plants. In addition they had the highest K^+/Na^+ ratio in their leaves. This may have contributed to their best salt tolerance.

The increased accumulation of K^+ and, at lesser extent, of Na^+ in plant tissues could lead to decreased cell osmotic potential, enhancing plant water uptake and maintaining leaf turgor under salt stress. In the present study, the bicistronic transgenic plants exhibited significantly higher leaf water retention than the single *TNHXSI* or *TVPI* transgenic lines under salt stress (Fig. 8c). In addition, the TP3 transgenic plants had higher chlorophyll content and greater biomass under salinity conditions.

1 This is probably due to a greater leaf turgor resulting from more balanced osmotic potential which
2 contributes to achieving higher plant photosynthesis and consequently improved growth in the TP3
3 plants compared to the single-transgenic plants (Table 1).

4 An important consequence of salinity stress is the generation of excessive reactive oxygen
5 species (ROS) which leads to cell toxicity, membrane dysfunction and cell death. Plants defend against
6 the ROS by enhancement of anti-oxidative enzymes. Catalase, which is involved in the degradation of
7 H₂O₂ into water and oxygen, is the major H₂O₂ scavenging enzyme in all aerobic organisms (Yang
8 and Poovaiah, 2002.). Catalase is critical for maintaining the redox balance during oxidative stress. It
9 was reported that salinity decreases CAT activity through stress-induced damage to the enzyme (Khedr
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11 salt-sensitive ones (Gossett et al. 1994). The over-expression of bacterial CAT gene conferred greater
12 salt tolerance to transgenic rice plants (Moriwaki et al. 2008). In this study, CAT activity was shown to
13 be higher in transgenic plants compared to wild type plants. Interestingly, the TP3 plants retained the
14 highest CAT activity under salt stress. The higher antioxidant capacity of the TP3 transgenic plants
15 may have contributed their less degree of cell membrane damage under salt stress (Fig. 8a and b).

16 Overall, our results demonstrated the usefulness of co-expression of the *TNHXS1* and the
17 *TVPI* genes using a dicistronic system involving IRES in the improvement of salt and drought
18 tolerance in transgenic plants. Also, our results seem to indicate that K⁺ accumulation and increased K⁺
19 transport are prerequisites for plant salt tolerance. Based on these encouraging results, we intend to
20 transform some economically important crops such as wheat and other crops using this bicistronic
21 vector. In addition, the preparation of different bicistronic constructs, involving potentially important
22 genes which confer drought and salinity tolerance to plants in combination with stress-inducible
23 promoters, is in progress.

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Figure legends

Figure 1. Salt and hygromycin tolerance of yeast cells harboring *TNHXS1* cDNA. **a.** saturated cultures of AXT3 transformants were diluted to an $OD_{600} = 0.8$, and 10 μ l of 10-fold serial dilutions were spotted onto YPG medium supplemented as follows: Control (no additions), NaCl (100 mM), KCl (1 M), LiCl (20 mM) and hyg (20 μ g/ml). **b.** the absorbance at 600 nm of yeast transformants is shown after shaking 48 h at 30 °C. The cells were grown in liquid YPG with or without 100 mM NaCl or 1 M KCl. Values are the average of three replicate samples. **(c, d)** Na^+ and K^+ content of cells grown in the presence of 100 mM NaCl, **(e, f)** Na^+ and K^+ content of cells grown in the presence of 1 M KCl. Values are the average of three replicate samples.

Figure 2. Molecular characterization of single *TNHXS1*, *TVPI* and TP3 transgenic plants. **a** Schematic structure of T-DNA region in plasmid pCambia2300-35S-*TNHXS1*, pCambia2300-35S-*TVPI* and pCambia2300-35S-*TNHXS1*-IRES-*TVPI*. RT-PCR analysis of **(b)** *TNHXS1*, **(c)** *TVPI* and **(d)** *TNHXS1* and *TVPI* expression in tobacco transgenic lines. Specific PCR products of 0.5 kbp (*TNHXS1*-Fw/*TNHXS1*-Rev) and 1.2 kbp (*TVP*-Fw/*TVP*-Rev) were amplified in *TNHXS1* (*TNHXS1*.cl3 and *TNHXS1*.cl17) and *TVPI* (*TVP*1cl19 and *TVP*1cl20) transgenic lines, respectively. The two transgenic lines co-expressing *TNHXS1* and *TVPI* (TP3cl38 and TPcl44) showed the PCR product of *TNHXS1* (*TNHXS1*-Fw/*TNHXS1*-Rev) and *TVPI* (*TVP*-Fw/*TVP*-Rev) as well as a specific product from the amplification using *TNHXS1* and IRES (*TNHXS1*-Fw/SP.2), IRES and *TVPI* (SP.1/*TVP*.2) and the full-length bicistronic (TN.1/*TVP*.2) specific primers. WT, Wild type. A 150 bp actin fragment was amplified by RT-PCR as an internal control.

Fig. 3. Activities of **(a)** H^+ -PPiase, **(b)** vacuolar H^+ -ATPase and **(c)** Na^+/H^+ antiport in tobacco tonoplast vesicles. Inside-acid pH gradients were established in vesicles purified from the roots of wild-type and transgenic plants (*TNHXS1*, *TVPI*, TP3) after the addition of pyrophosphate or ATP. Proton pumping by the H^+ -PPiase or V-ATPase was calculated from initial rates of fluorescence decline. Once fluorescence was stabilized, 100 mM of NaCl were added, and fluorescence recovery indicating cation/proton exchange was monitored for 2 min. In **(d)**, the cation antiport activity is represented when assayed either with NaCl or KCl at the indicated concentrations. Activities are expressed in arbitrary

units as the relative change in fluorescence ($\Delta F/F_{\max}$) per minute and milligram of membrane protein. Values are means of three independent biological replicates. On top of bars, the standard error of the mean is represented.

Fig. 4. Growth analysis of TP3 and single transgenic lines at seedling stage. Seeds were germinated on MS medium supplemented with Kanamycin and transferred to MS supplemented with various concentrations of NaCl. Phenotype of WT and transgenic lines grown on MS (a), 100 mM (b) and 200 mM (c) NaCl for 45 days. d Root length. e Fresh weight. f Dry weight. Values are means $\pm$ S.D (n = 5-10). Values having the same letter in each NaCl concentration are not significantly different at $P < 0.05$, by Duncan's MRT.

Fig. 5 Sodium and potassium content in leaf and root of WT, single *TNHXS1*, *TVPI* and TP3 transgenic seedlings under different NaCl concentrations. The Na^+ (a, b) and K^+ (c, d) contents were measured after 45 days of salt treatment. Values are means $\pm$ S.E (n = 5). Values having the same letter in each NaCl concentration are not significantly different at $P < 0.05$, by Duncan's MRT.

Fig. 6 Growth analysis of WT, single *TNHXS1*, *TVPI* and TP3 transgenic plants under normal and 250 mM NaCl concentrations in greenhouse. Phenotypes of WT and transgenic plants at absence of salt treatment (a) and irrigated continuously with 250 mM NaCl solution (b). Root growth from non stressed plants (c) and plants exposed continuously to 250 mM NaCl (d). Percentage reduction (%) compared with unstressed control (e).

Fig. 7 Effect of salt stress on chlorophyll content. a Leaf disc senescence assay for salinity tolerance in transgenic tobacco plants grown under greenhouse condition: representative picture to show phenotypic differences in leaf discs. b Chlorophyll content from leaves of WT and different transgenic seedlings grown *in vitro* in MS medium supplemented with 100 and 200 mM NaCl solution. Values are means $\pm$ SE (n = 5). Values having the same letter in each NaCl concentration are not significantly different at ($p < 0.05$), by Duncan MRT.

1 **Fig. 8** Effect of salt stress on catalase activity, electrolyte leakage and relative water content in
2 transgenic (monotransgenic and TP3) and WT plants under greenhouse-based salt stress. **a** Catalase
3 activity. **b** Electrolyte leakage of leaf cells and. **c** Relative water content (RWC). Values are means $\pm$
4 SE (n = 5). Values having the same letter in each NaCl concentration are not significantly different at
5 (p < 0.05), by Duncan MRT.

Table 1

Table 1 Comparison of different growth parameters and seed production of the wild type, single *TVP1*, *TNHXS1* transformants and TP3 transgenic plants grown in the absence or the presence of 250 mM NaCl for 45 days under greenhouse conditions.

Parameter	Control					Salt stress (250 mM NaCl)								
	WT	TNHXS cl3	TNHXS1cl7	TVP1cl19	TVP1cl20	TP3cl38	TP3cl44	WT	TNHXS cl3	TNHXS1cl7	TVP1cl19	TVP1cl20	TP3cl38	TP3cl44
Plant weight (gm)	116.2 (5a)	138.8 (2.3b)	141.8 (1.8b)	159.9 (2.7d)	154.6 (3.1c)	160.3 (1.8d)	161.3 (5.d)	22 (2.5a)	53.5 (4.7b)	52 (2.2b)	71.1 (3d)	72.3 (3.4d)	73.6 (2.9d)	62.6 (3.9c)
Shoot height (cm)	51.6 (2a)	62 (5bc)	65 (3.1bc)	66.2 (8.5c)	66.6 (4.9c)	57 (2.9ab)	62.2 (4bc)	12.3 (1.5a)	23 (2b)	21 (2b)	32.3 (1.5d)	34.6 (2.0d)	33.8 (2.0c)	29.2 (1.7c)
Root length (cm)	14.6 (0.7a)	14.8 (0.7a)	16.6 (0.4b)	20.8 (1.3c)	23.9 (0.7d)	25.1 (1.1d)	34.2 (0.5e)	6.8 (0.7a)	11.5 (0.5b)	12.1 (1.0b)	13.8 (0.2c)	16 (1.3d)	16 (0.7d)	16 (1.0d)
No. of leaves per plant	40 (2a)	48.3 (2.0b)	48 (3.6b)	46.2 (2.9ab)	42 (6.9ab)	47.5 (2.3b)	45.8 (5ab)	12.6 (2.0a)	23.6 (1.5b)	22.7 (2.8b)	30.6 (3.0cd)	30 (1.7c)	36.2 (6d)	42.2 (1.8e)
No. of pods per plant	21.3 (2a)	22 (1a)	30.2 (1.0b)	22.2 (2.1a)	21.6 (2.4a)	18 (1.4a)	33.2 (3b)	0 (a)	1(ab)	2.2 (bc)	4.3 (c)	2 (ab)	3 (bc)	3 (bc)

Values are means ± SE (n = 8). Values having the same letters in each NaCl concentration are not significantly different at (p < 0.05), by Duncan MRT.

Figure 4

Figure 6

Figure 7