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Co-expression of xerophyte *Zygophyllum xanthoxylum ZxNHX* and *ZxVP1-1* enhances salt and drought tolerance in transgenic *Lotus corniculatus* by increasing cations accumulation

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Abstract. *Lotus corniculatus* L. is an important legume for forage, but is sensitive to salinity and drought. To develop salt- and drought-resistant *L. corniculatus*, *ZxNHX* and *ZxVP1-1* genes encoding tonoplast Na^+/H^+ antiporter and H^+-pyrophosphatase (H^+-PPase) from a succulent xerophyte *Zygophyllum xanthoxylum* L., which is well adapted to arid environments through accumulating Na^+ in its leaves, were transferred into this forage. We obtained the transgenic lines co-expressing *ZxNHX* and *ZxVP1-1* genes (VX) as well as expressing *ZxVP1-1* gene alone (VP). Compared with wild-type, both VX and VP transgenic lines grew better at 200 mM NaCl, and also exhibited higher tolerance and faster recovery from water-deficit stress: these performances were associated with more Na^+, K^+ and Ca^{2+} accumulation in their leaves and roots, which caused lower leaf solute potential and thus retained more water. Moreover, the transgenic lines maintained lower relative membrane permeability and higher net photosynthesis rate under salt or water-deficit stress. These results indicate that expression of tonoplast Na^+/H^+ antiporter and H^+-PPase genes from xerophyte enhanced salt and drought tolerance of *L. corniculatus*. Furthermore, compared with VP, VX showed higher shoot biomass, more cations accumulation, higher water retention, lesser cell membrane damage and higher photosynthesis capacity under salt or water-deficit condition, suggesting that co-expression of *ZxVP1-1* and *ZxNHX* confers even greater performance to transgenic *L. corniculatus* than expression of the single *ZxVP1-1*.

Additional keywords: H^+-PPase, tonoplast Na^+/H^+ antiporter.

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Introduction

Salinity and drought are two of the major environmental factors limiting the development, productivity and quality of crops worldwide, since most crops are sensitive to salinity and drought (Zhu 2002). To cope with salt stress, plants have evolved a series of adaptation strategies: one of these is compartmentalising Na^+ into the vacuole, which provides an efficient way not only to alleviate the toxic effects of excess Na^+ in cytosol but also to maintain cell osmotic potential by using Na^+ as a cheap osmoregulation substance, which drives water uptake into cells (Apse *et al.* 1999; Gaxiola *et al.* 2001, 2007; Bao *et al.* 2009; Gouiaa *et al.* 2012). Na^+ compartmentalisation is mediated by a tonoplast Na^+/H^+ antiporter, which is driven through proton motive force across tonoplast generated by the tonoplast H^+-pumps, H^+-ATPase and H^+-pyrophosphatase (H^+-PPase) (Apse *et al.* 1999; Gaxiola *et al.* 2002; Bao *et al.* 2009). Theoretically, expression of tonoplast Na^+/H^+ antiporter and H^+-PPase genes should increase the sequestration of ions in the vacuole, thus, alleviating toxicity of Na^+ in the cytosol and enhancing vacuolar osmoregulatory capacity. This should, in turn, confer salt and drought tolerance to the plant.

Indeed, previous studies on the overexpression of the genes encoding Na^+/H^+ antiporters from different sources have demonstrated that there are enhanced salt and drought tolerance in various transgenic plants, including *Arabidopsis* (Apse *et al.* 1999; Brini *et al.* 2007), wheat (Xue *et al.* 2004), cotton (He *et al.* 2005), rice (Chen *et al.* 2007; Liu *et al.* 2010), tomato (Zhang and Blumwald 2001; Bhaskaran and Savithramma 2011) and *Brassica napus* L. (Zhang *et al.* 2001). Genetic manipulation to improve plant salt and drought resistance has also been achieved by overexpressing tonoplast H^+-PPase genes in transgenic plants of *Arabidopsis* (Gaxiola *et al.* 2001), tobacco (Gao *et al.* 2006), tomato (Park *et al.* 2005), cotton (Lv *et al.* 2008; Pasapula *et al.* 2011), creeping bentgrass (Li *et al.* 2010) and alfalfa (Bao *et al.* 2009). It has been demonstrated that co-expression of the *Suaeda salsa SsNHX1* and *Arabidopsis*

AVP1 confers higher salt tolerance than expression of the single *SsNHX1* in transgenic rice (Zhao *et al.* 2006). Recently, Bhaskaran and Savithramma (2011) reported the similar findings in transgenic tomato co-expressing *Pennisetum glaucum PgNHX1* and *AVP1*. Gouiaa *et al.* (2012) co-transferred wheat *TNHXS1* and *TVP1* into tobacco, and proposed that co-ordinating expression of these two genes is more valuable way for obtaining transgenic plants with enhanced stress tolerance. However, so far the tonoplast Na^+/H^+ antiporter and H^+-PPase genes used for improvement of abiotic stress resistance are mainly from model plants, crops and halophytes.

Zygophyllum xanthoxylum L. is a succulent xerophyte that is widely distributed in desert regions of northwestern China, and is recognised as one of the most drought-resistant plants known (Liu *et al.* 1987). Our previous studies showed that *Z. xanthoxylum* could accumulate larger quantities of Na^+ through roots, and transport Na^+ to leaves for osmotic adjustment even at low soil salt, suggesting that Na^+ may have a positive function in the response of *Z. xanthoxylum* to water stress (Wang *et al.* 2004). Yue *et al.* (2012) found that 5–100 mM NaCl in the growth medium significantly increased the growth of *Z. xanthoxylum*, and the optimal growth was obtained at 50 mM NaCl. Furthermore, the addition of 50 mM NaCl significantly alleviated the deleterious impact of osmotic stress on the growth of *Z. xanthoxylum*. Ma *et al.* (2012) demonstrated that the contribution of Na^+ to the total osmotic potential varied from 8% in the control to 13% in plants subjected to water-deficit and further to 28% in plants grown in the presence of 50 mM NaCl under water deficit; however, the contribution of K^+ significantly decreased from 13 to 8%. These findings suggest that Na^+ plays an important role in osmotic adjustment in *Z. xanthoxylum* under water deficit. Therefore, we cloned the genes encoding *Z. xanthoxylum* tonoplast Na^+/H^+ antiporter and H^+-PPase, *ZxNHX* (GenBank accession number: EU103624) and *ZxVP1-1* (GenBank accession number: EU103625), and found that there was a positive correlation between upregulation of *ZxNHX* and Na^+ accumulation in leaves of *Z. xanthoxylum* treated with salt or drought, indicating that ZxNHX is involved in Na^+ compartmentation in leaf (Wu *et al.* 2011). However, little is known about the effectiveness when *ZxNHX* and *ZxVP1-1* are introduced into important crops, especially, forage legumes.

Lotus corniculatus L. is not only one of the most important legume forages with high protein content and low saponin level, but is also a model plant among forage legumes, with best reproducibility. However, most *L. corniculatus* cultivars are sensitive to salt and drought (Cheng *et al.* 2010). Therefore, breeding the *L. corniculatus* cultivars with enhanced salt and drought resistance is necessary for this important forage to adapt to saline and arid soils. But a considerable amount of time is required to select salt and drought tolerant plants via conventional breeding process. Transgenetic technologies have provided an efficient way for improving *L. corniculatus*. Thus, in this study, we investigated the possibility that expressing *Z. xanthoxylum ZxNHX* and *ZxVP1-1* enhances salt and drought tolerance in *L. corniculatus*, and compared the stress-resistance between transgenic *L. corniculatus* lines expressing single *ZxVP1-1* and co-expressing both *ZxNHX* and *ZxVP1-1*. The resulting transgenic lines exhibited enhanced salt and drought tolerance than the wild-type, and especially we noted that co-expression of the *ZxNHX* and *ZxVP1-1* was superior to expression of the single *ZxVP1-1* for protecting transgenic *L. corniculatus* from high salinity and drought damage.

Materials and methods

Plasmid construction and bacterial strains

The plasmid pCAMBIA1302-*ZxVP1-1* (12.1 kb) and pCAMBIA1302-*ZxNHX-ZxVP1-1* (15.3 kb) have been previously constructed in our laboratory (J-J Xi and S-M Wang, unpubl. data). These two plasmids contain the cauliflower mosaic virus 35S (CaMV 35S) promoter driving the *Zygophyllum xanthoxylum* L. *ZxNHX* (GenBank accession number: EU103624, encoding tonoplast Na^+/H^+ antiporter) and/or *ZxVP1-1* (GenBank accession number: EU103625, encoding tonoplast H^+-PPase) genes (Wu *et al.* 2011) amplified from cDNA, and the *HPT II* gene for hygromycin resistance as the selectable marker (see Fig. S1, available as Supplementary Material to this paper). The constructs were mobilised into *Agrobacterium tumefaciens* strain GV3101 by electroporation for subsequent plant transformation respectively.

Plant material and transformation

Lotus corniculatus L. (cv. Mirabel) cotyledons as explants were used for *A. tumefaciens*-mediated transformation as described by Cheng *et al.* (2010) with minor modifications. Briefly, the transformed explants and plantlets were screened on B_5 medium (Gamborg *et al.* 1968) contained 0.5 mg L^{-1} 6-BA supplemented with 15 mg L^{-1} hygromycin. After 4 weeks, the resistant shoots were separated and transferred to half-strength MS medium (Murashige and Skoog 1962) supplemented with 0.05 mg L^{-1} NAA for initiation of rooting. Finally, the regenerated resistant plants were transferred into soil and cultured in the greenhouse under a photoperiod 16 h/8 h (light/dark) with supplemental lighting at 28 °C in the light and 24 °C in the dark. The wild-type (WT) plants were originated from the same tissue culture process that the transgenic plants were produced.

Molecular characterisation of transgenic L. corniculatus

For PCR analysis, plant genomic DNA was isolated from 0.2 g of young leaf material of putative transformants and WT plants according to the cetyltrimethyl ammonium bromide (CTAB) method (Bao *et al.* 2009). The transgenic plants were confirmed by PCR amplification of a 471-bp fragment of the *ZxVP1-1* gene and a 504-bp fragment of the *ZxNHX* gene respectively. The two primers used for *ZxVP1-1* amplification were P1 (5′-GCTGGAATCGAATTTGTGGA-3′) and P2 (5′-TGCAGCCTTATGTGCATCTG-3′), whereas those for *ZxNHX* amplification were P3 (5′-CATCGGTGGTGCTTTTCAAT-3′) and P4 (5′-GCAGCTCTACCAGCCATCAC-3′). The PCR amplification conditions for the *ZxVP1-1* and *ZxNHX* fragments were an initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94°C for 1 min, 54°C for 30 s, 72°C for 30 s, then a final extension at 72°C for 5 min. PCR products were electrophoresed on a 1.2% agarose gel containing ethidium bromide and visualised by AlphaImager (ver. 4.0.1; Alpha

Innotech Co., San Leandro, CA, USA) for subsequent analysis respectively. Experiments were repeated at least three times.

For RT-PCR analysis, total RNA was extracted from 0.2 g of young leaf material of PCR-positive plants and WT plants with the guanidine of the EZ-10 Spin Column Total RNA Isolation Kit (Sangon, Shanghai, China), and then were treated with RNase-free DNase to remove contaminating DNA. DNase-treated RNA samples were reverse-transcribed using M-MuLV reverse transcription kit (Sangon, Shanghai, China) to synthesise the first strand cDNA. The reverse transcribed cDNA samples were used for semiquantitative PCR, the specific fragments of *ZxVP1-1* (471 bp) and *ZxNHX* (504 bp) were amplified with two pairs of primers P1 and P2, P3 and P4 respectively. *ACTIN* gene was used as the internal control in the semiquantitative RT-PCR. The specific primers of *ACTIN* that amplified a 598-bp fragment are P5 (5′-GTGGTCGTACAACAGGTATTGTG-3′) and P6 (5′-GAACCTCCAATCCAGACACTG-3′). The PCR condition for *ACTIN* was performed as follows: 94°C for 2 min; 25 cycles of 94°C for 30 s, 56°C for 45 s and 72°C for 50 s; and a final extension at 72°C for 8 min. The amplified fragments were separated on 1.2% (w/v) agarose gel containing ethidium bromide and visualised by AlphaImager for subsequent analysis. Experiments were repeated at least three times.

Salt and water-deficit treatments

To prepare the materials for physiological analysis, the T_0 transgenic lines and WT plants were propagated from stem cutting (Bao *et al*. 2009; Cheng *et al*. 2010). The stem sections with 2–3 nodes were excised using a sharp razor blade and placed the base into moist vermiculite and perlite (1 : 1) under a photoperiod of 16 h/8 h (light/dark) at 24 ± 2°C and 90 ± 5% RH in the growth chamber. After 2 weeks, when the adventitious roots had formed, plants with the same size shoots and roots were chosen for further culture.

For salt treatments, the transgenic *L. corniculatus* and WT plants were transferred to plastic culture pots (cylindrical pot with 8 cm in diameter and 10 cm in height, one plant per pot) containing vermiculite and perlite (1 : 1) under a photoperiod of 16/8 h (light/dark, the light flux density during the light period was a~1000 μmol m^{-2} s^{-1}) at 26 ± 2°C and 60 ± 5% RH, watered every 2 days with 1/2 strength Hoagland nutrient solution for 4 weeks, and then the nutrient solution was supplemented with NaCl, increasing with 50 mM day^{-1} increments to final concentrations (0, 100 and 200 mM). After salt treatment for 10 days, the plants were used for further physiological tests.

For water-deficit treatments, plants from *L. corniculatus* transgenic lines and WT were transplanted to plastic culture pots (cylindrical pot with 8 cm in diameter and 10 cm in height, one plant per pot) containing artificial soil (vermiculite : perlite : peat moss equal to 1 : 1 : 1) under a photoperiod of 16 h/8 h (light/dark, the light flux density during the light period was ~1000 μmol m^{-2} s^{-1}) at 26 ± 2°C and 60 ± 5% RH, watered every 2 days with 1/8 strength Hoagland nutrient solution to field water capacity for 4 weeks, then the water was withheld until all the plants were wilting. During water-deficit treatment, physiological indicators were measured with intervals of 3 days.

Measurement of biomass and cations concentrations

The concentrations of Na^+, K^+ and Ca^{2+} in leaves and roots of transgenic and WT plants were measured according to the method by Bao *et al.* (2009). Roots were firstly rinsed with deionised water for 10 s, and then washed with 20 mM cold $LiNO_3$ solution. The leaves and roots were dried at 80°C for 48 h, the DW was measured. The cations were extracted from dried plant tissues in 100 mM acetic acid at 90°C for 2 h, then the concentrations of Na^+, K^+ and Ca^{2+} were determined using a flame spectrophotometer (No. 2655-00, Coleparmer Instrument Co, Vernon Hills, IL, USA).

Measurement of the solute potential and the relative water content

The solute potential (ψs) was detected following the method described by Gaxiola *et al.* (2001). Leaves from transgenic and WT plants of each treatment were rinsed with deionised water and blotted on filter paper immediately, then were frozen in liquid nitrogen and thawed to extrude sap by a syringe. The resulting sap was determined with a cryoscopic osmometer (OSMOMAT-030, GONOTEC GmbH, Munich, Germany). The readings (mmol kg^{-1}) were used to calculate the solute potential in MPa with the following formula: ψs = –moles of solute × RK, where $r = 0.008314$ and K = 298°C.

The FW of *L. corniculatus* leaves were weighed immediately after being excised from plants. Then the leaves were soaked in deionised water at 4°C overnight, and their rehydrated weights were determined. Finally, they were dried in an oven at 80°C for 48 h and weighed. The RWC was calculated as indicated: RWC = (FW – DW)/(rehydrated weight – DW).

*Measurement of the relative membrane permeability (RMP) and the net photosynthetic rate (*Pn*)*

The RMP of leaf cells was measured according to work by Bao *et al.* (2009) using a conductivity meter (EC215, Hanna, Woonsocket, RI, USA). RMP was calculated using the following equation: RMP (%) = S1/S2 × 100, where S1 and S2 refer to conductivity of *L. corniculatus* live leaves and boiled leaves respectively.

Pn was measured by an automatic photosynthetic measuring apparatus (LI-6400, Li-Cor Biosciences, Lincoln, NE, USA). Leaf area was estimated by the Epson Perfection 4870 photo scanner (Epson America Inc., Long Beach, CA, USA).

Statistical analysis

Data for this study were subjected to one-way analysis of variance (ANOVA) using SPSS 16.0 (SPSS Inc., Chicago, IL, USA) and Duncan's multiple range tests were used to detect significant differences between means at a significance level of $P < 0.05$. All data were presented as means ± s.e.

Results

Production and molecular characterisation of transgenic L. corniculatus *plants co-expressing both* ZxNHX *and* ZxVP1-1 *and expressing* ZxVP1-1

To investigate the potential benefit of transferring tonoplast Na^+/H^+ antiporter gene *ZxNHX* and H^+-PPase gene *ZxVP1-1*

from xerophyte *Z. xanthoxylum*, we identified a total of 15 and seven independent *ZxNHX+ZxVP1-1* and *ZxVP1-1* transgenic *L. corniculatus* lines through PCR amplification respectively (Fig. S2). The expression levels of *ZxNHX* and *ZxVP1-1* were detected by RT-PCR in young leaves of all transgenic lines and WT plants respectively. As expected, *ZxNHX* and *ZxVP1-1* co-expressed in all 15 *ZxNHX+ZxVP1-1* transgenic lines (1–15), which were observed in two separate experiments with *ZxNHX* and *ZxVP1-1*-specific primers, respectively; the levels of the same gene varied between different lines, and all of *ZxVP1-1* and *ZxNHX* showed the highest expression level in line 1, as well as, the lowest expression level in line 15 (Fig. 1*a*). Similarly, *ZxVP1-1* expression was detected in all 7 *ZxVP1-1* transgenic lines (16–22), and line 22 and line 16 showed the highest and lowest *ZxVP1-1* expression level respectively (Fig. 1*b*).

Expressions of ZxNHX *and* ZxVP1-1 *improve the salt and drought tolerance in transgenic* L. corniculatus

The same size plants were cultured in non-stress condition for 4 weeks, then 10 transgenic lines co-expressing *ZxNHX* and *ZxVP1-1* (lines1, 2, 3, 6, 8, 9, 10, 12, 13, 15), and five transgenic lines expressing *ZxVP1-1* (lines16, 18, 20, 21, 22) were treated with 200 mM NaCl for 10 day and withheld water for 10 day respectively. All of these transgenic lines showed higher plant height and more shoot biomass than wild-type, suggesting they might possess enhanced resistance to salt and drought (see Table S1, available as Supplementary Material to this paper). To investigate the salt and drought tolerance of transgenic *L. corniculatus* in detail, line 1 (now called VX) and line 22 (now called VH) with highest gene(s) expression level were chosen for further physiological assays.

After grown in non-stress condition for 4 weeks, both transgenic and WT plants grew vigorously, but the transgenic plants developed faster to larger size than WT plants (Fig. 2*a*). When plants were exposed to 200 mM NaCl for 10 days, WT plants displayed chlorosis and a general growth inhibition, whereas both VX and VH transgenic grew well (Fig. 2*b*). The shoot DW of all plants decreased progressively with external NaCl concentration increasing, but that of WT plants was significantly lower than that of transgenic plants under the same NaCl concentration (Fig. 2*c*). For instance, in the presence of 200 mM NaCl, the shoot DW of VX and VH were 3.7-fold and 3.3-fold of that of WT plants respectively (Fig. 2*c*). Although shoot biomass has no any significant differences between two transgenic lines under non-stress condition and 100 mM NaCl, the shoot DW of VX is 11% higher than that of VH plants at 200 mM NaCl (Fig. 2*c*).

Four-week-old plants were withheld water for 10 days, the WT plants showed growth inhibition and chlorosis, whereas the transgenic lines continued normal growth (Fig. 3*a*) until withholding water for 14 days when all plants wilted (Fig. 3*b*). Then the plants were rewatered for 4 days, WT plants showed permanent wilting and finally died, but both transgenic lines recovered and survived from temporary wilting (Fig. 3*c*, *d*). During water-deficit treatment, the growth of transgenic lines was significantly faster than that of wild-type. After withholding water for 12 days, the increments of shoot DW in transgenic lines VX and VH were 65 and 55%, respectively, whereas it was only 39% in WT plants. Furthermore, the performance of VX also was superior to VH during water-deficit treatment, and the shoot DW of VX was 10% higher than that of VH after withholding water for 12 days (Fig. 3*e*), which is similar to the situation under salt stress.

Transgenic L. corniculatus *lines accumulate more* Na^+, K^+ *and* Ca^{2+}

As demonstrated in Fig. 4, both transgenic lines accumulated more Na^+, K^+ and Ca^{2+} in leaves and roots than WT plants even when they grew in normal condition (no NaCl application), and it was also observed that VX retained more cations in its leaves or roots than VH under either normal condition or NaCl treatments.

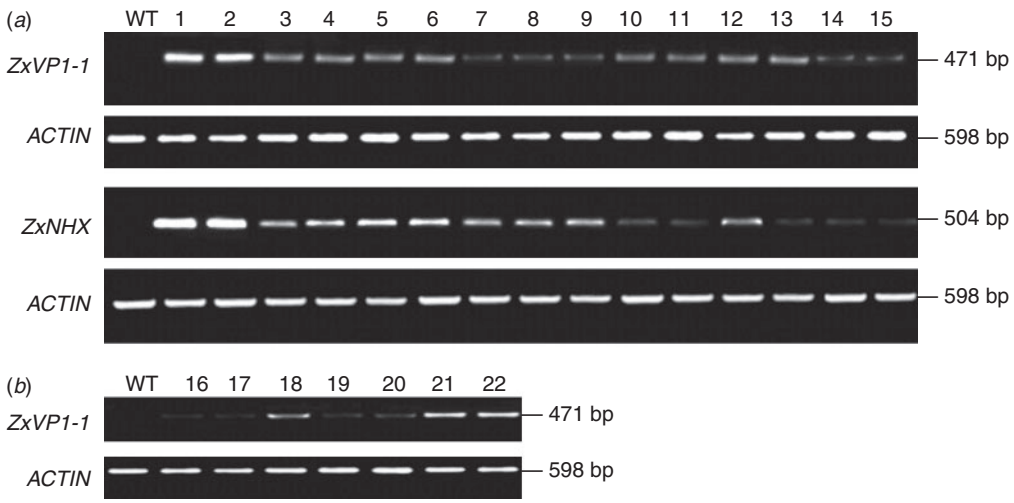

Fig. 1. PT-PCR analysis of (*a*) *ZxNHX* and *ZxVP1-1*, and (*b*) *ZxVP1-1* expression in transgenic *Lotus corniculatus*. Specific PCR products (471 bp for *ZxVP1-1*, 504 bp for *ZxNHX*) were detected in transgenic *L. corniculatus*. WT, wild-type plant; 1–15, transgenic lines co-expressing *ZxNHX* and *ZxVP1-1*; 16–22, transgenic lines expressing *ZxVP1-1*. A 598-bp *ACTIN* gene fragment was amplified by RT-PCR as an internal control.

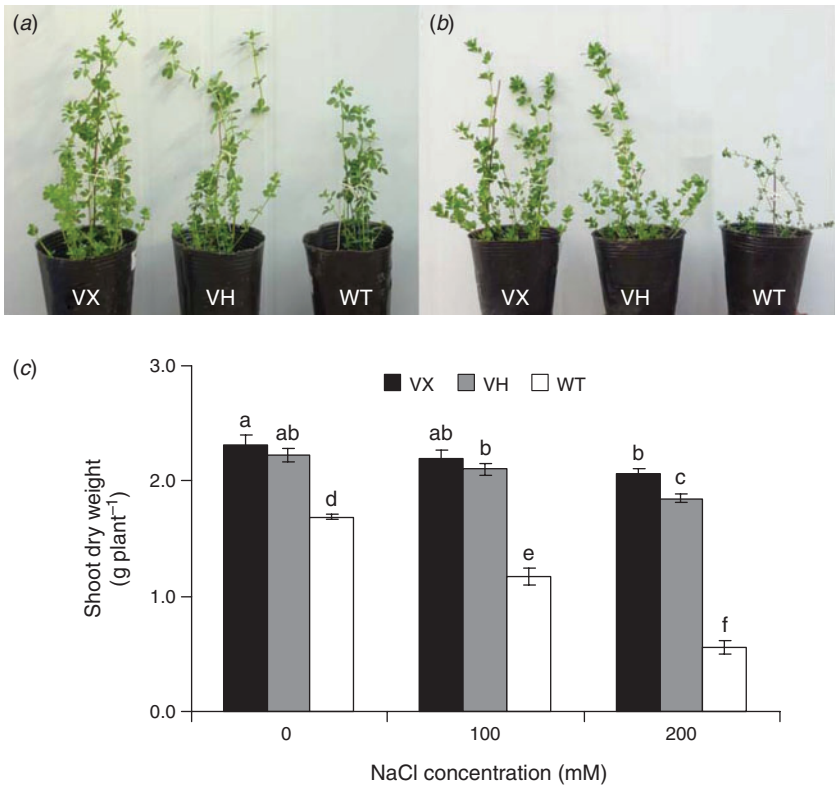

Fig. 2. Transgenic *Lotus corniculatus* plants showed enhanced salt tolerance. Plants were watered every 2 days with 1/2 Hoagland nutrient solution for 4 weeks, and then the nutrient solution was supplemented with NaCl, incrementally increasing with 50 mM day^{-1} increments to final concentrations (0, 100 and 200 mM). The photographs show representative plants of two transgenic lines and wild-type at the 10th day without (*a*) and with 200 mM NaCl (*b*). (*c*) Shoot DW of transgenic and wild-type *L. corniculatus* under different NaCl concentrations for 10 days. Data are means ± s.e. ($n = 12$). Columns with different letters indicate significant difference at $P < 0.05$ (Duncan's test). WT, wild-type plants; VX, transgenic line co-expression *ZxNHX* and *ZxVP1-1*; VH, transgenic line expressing *ZxVP1-1*.

With external NaCl increasing, Na^+ concentrations significantly increased in the leaves and roots of two transgenic lines and wild-type, but the increases in VX and VP transgenic plants were much higher than that in WT plants (Fig. 4*a*, *b*). The amounts of K^+ dropped gradually in leaves and roots of both transgenic lines and WT plants with external NaCl increasing, but a greater decrease was observed in the latter (Fig. 4*c*, *d*). Under salt treatments, Ca^{2+} concentrations significantly increased in leaves and roots of two transgenic lines but did not exhibit obvious change in WT plants (Fig. 4*e*, *f*).

With the increase of water-deficit treatment time from 0 to 12 days, all of three cation concentrations in leaves and roots of both VX and VH significantly increased, although they did not show obvious changes in WT plants, leading to more Na^+, K^+ and Ca^{2+} accumulation in transgenic plants (Fig. 5). Like the situation as NaCl treatments, VX accumulated more Na^+, K^+ and Ca^{2+} in its leaves and roots than VH during water-deficit treatment. For example, after the 12 days of water deficit, the Na^+, K^+ and Ca^{2+} concentrations in leaves of VX were 30, 39 and 22% higher than that in VH, respectively, and also 25, 20 and 40% higher in roots than that in VH respectively (Fig. 5).

Transgenic L. corniculatus *lines retain more solutes and water*

To assay the amount of solutes in the leaves, the leaf solute potentials (ψs) of wild-type and both transgenic lines were measured. When challenged with salt or water-deficit, the leaf ψs of transgenic and WT plants dropped significantly. However, under the same treatment, the leaf ψs of transgenic lines were lower than that of wild-type (Fig. 6*a*, *b*). Moreover, the leaf ψs of VX was more negative than that of VH under stress conditions (Fig. 6*a, b*).

In order to further study the osmoregulatory capacity of transgenic *L. corniculatus*, the leaf relative water contents (RWCs) were evaluated during salt or water-deficit treatment. Comparatively, the RWC of WT plants decreased by 63% under 200 mM NaCl, whereas the VX and VH dropped by only 18 and 24% respectively (Fig. 6*c*). Similarly, after 12 days of water-deficit stress, the RWC of WT plants was reduced by 61%, whereas the VX and VH only by 22 and 27% respectively (Fig. 6*d*). These results indicated that under salt or water-deficit conditions the expression of *ZxNHX* and *ZxVP1-1* caused more water retention in the leaves of transgenic plants

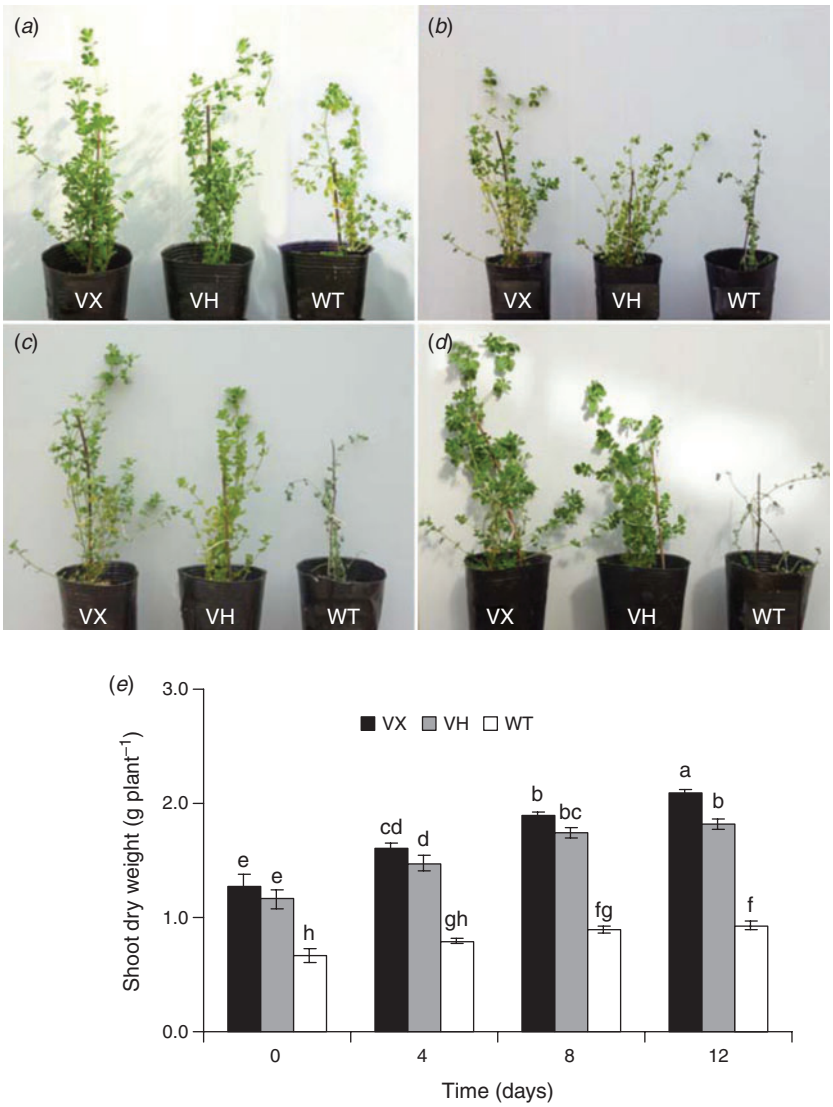

Fig. 3. Transgenic *Lotus corniculatus* plants showed enhanced drought tolerance. Representative plants of wild-type and two transgenic lines were watered with 1/8 Hoagland nutrient solution every 2 days to field water capacity for 4 weeks. After that, the soil (vermiculite : perlite : peat moss = 1 : 1 : 1) was allowed to dry by withholding water for 10 days (*a*), and 14 days (*b*) when all plants were wilting. Then soil was rewatered to field water capacity for 1 day (*c*), and 4 days (*d*). (*e*) Shoot DW of transgenic and wild-type plants during withholding water for 0, 4, 8 and 12 days respectively. Data are means ± s.e. ($n = 12$). Columns with different letters indicate significant difference at $P < 0.05$ (Duncan's test). WT, wild-type plants; VX, transgenic line co-expression *ZxNHX* and *ZxVP1-1*; VH, transgenic line expressing *ZxVP1-1*.

through improving their osmotic adjustment capacity; co-expression of both *ZxVP1-1* and *ZxNHX* confers a greater water retention capacity than expression of single *ZxVP1-1*.

Expressions of ZxVP1-1 *and* ZxNHX *maintain the stability of cell membrane and protect the photosynthetic machinery in transgenic* L. corniculatus

To investigate the stability of membrane in transgenic and WT plants challenged by salt and water-deficit treatments, relative membrane permeability (RMP) was measured. With the increase of salt concentration or water-deficit over time, the RMP increased significantly in all of plants, but showed a much greater increase in wild-type than in VX and VH lines (Fig. 7*a*, *b*). In transgenic lines VX and VH, the RMP was 24 and 30% under 200 mM NaCl (Fig. 7*a*), and was 23 and 28% after 12 days of water-deficit (Fig. 7*b*), respectively, significantly less than that in WT plants (57% at 200 mM NaCl and 46% at the 12 days of water-deficit respectively). These results suggest that the cell membranes of transgenic *L. corniculatus* plants are healthier and subjected to less damage under salt or drought stress.

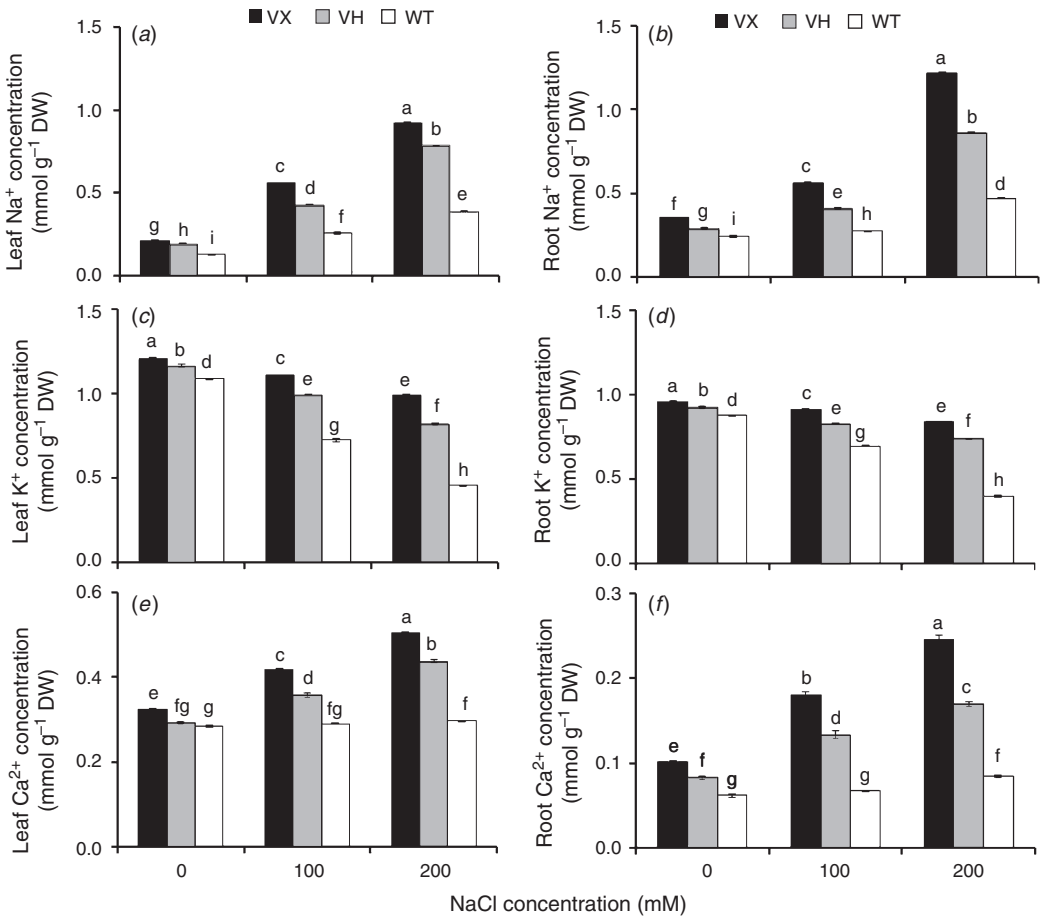

Fig. 4. Cation concentrations in leaf (left) and root (right) of transgenic and wild-type *Lotus corniculatus* under different NaCl concentrations. The Na^+ (*a*, *b*), K^+ (*c*, *d*), and Ca^{2+} (*e*, *f*) concentrations were determined after 10 days of salt treatments. Data are means ± s.e. ($n = 6$). Columns with different letters indicate significant difference at $P < 0.05$ (Duncan's test). WT, wild-type plants; VX, transgenic line co-expression *ZxNHX* and *ZxVP1-1*; VH, transgenic line expressing *ZxVP1-1*.

The increased salt and drought tolerance of VX and VH was further confirmed by measuring the net photosynthetic rate (*Pn*). The *Pn* declined in all plants under either salt or water-deficit treatments, but the remarkable decreases were observed in WT plants (Fig. 7*c*, *d*). After exposure to 200 mM NaCl for 10 days, the *Pn* was reduced by 58% in WT plants, but by only 29 and 39% in VX and VH respectively (Fig. 7*c*); after suffering from 12 days of water-deficit, the *Pn* of WT plants was reduced by 59%, but only 31 and 37% in VX and VH respectively (Fig. 7*d*).

Discussion

Expressions of ZxNHX *and* ZxVP1-1 *confer enhanced salt and drought tolerance and improve plant development in transgenic* L. corniculatus

Breeding cultivars with increased stress tolerance is one of the most efficient and economic strategies to cope with the challenges from salinity and drought-prone environments. Plants resistant to salt stress can be achieved through sequestrating Na^+ into vacuole (Flowers 2004; Yamaguchi and Blumwald 2005; Munns and Tester 2008). Many previous studies have demonstrated that expressing tonoplast Na^+/H^+ antiporter or/and H^+-PPase led to enhanced salt- and drought-tolerance in various plant species (Apse *et al.* 1999; Gaxiola *et al.* 2001; Zhang and Blumwald 2001; He *et al.* 2005; Gao *et al.* 2006; Brini *et al.* 2007; Bao *et al.* 2009; Li *et al.* 2010; Pasapula *et al.* 2011). A large number of genes encoding tonoplast Na^+/H^+ antiporter and H^+-PPase were used in different works, but there is no work published estimating the functions of homologous genes from xerophyte species. In the present work, the *ZxNHX* and *ZxVP1-1* genes from xerophyte *Z. xanthoxylum* were firstly introduced into the important legume forage *L. corniculatus*. We obtained transgenic lines co-expressing both *ZxNHX* and *ZxVP1-1*, as well as expressing single *ZxVP1-1* (Fig. 1), and found that both types of transgenic *L. corniculatus* showed enhanced salt and drought tolerance. After treatment with high salt concentration or water deficit, transgenic plants survived and recovered quickly, whereas WT plants displayed progressive chlorosis, reduced biomass, a general growth inhibition, and even death (Figs 2, 3). These results indicate that *ZxNHX* and *ZxVP1-1* from the xerophyte *Z. xanthoxylum* are likely candidate genes for enhancing salt and drought tolerance of crops.

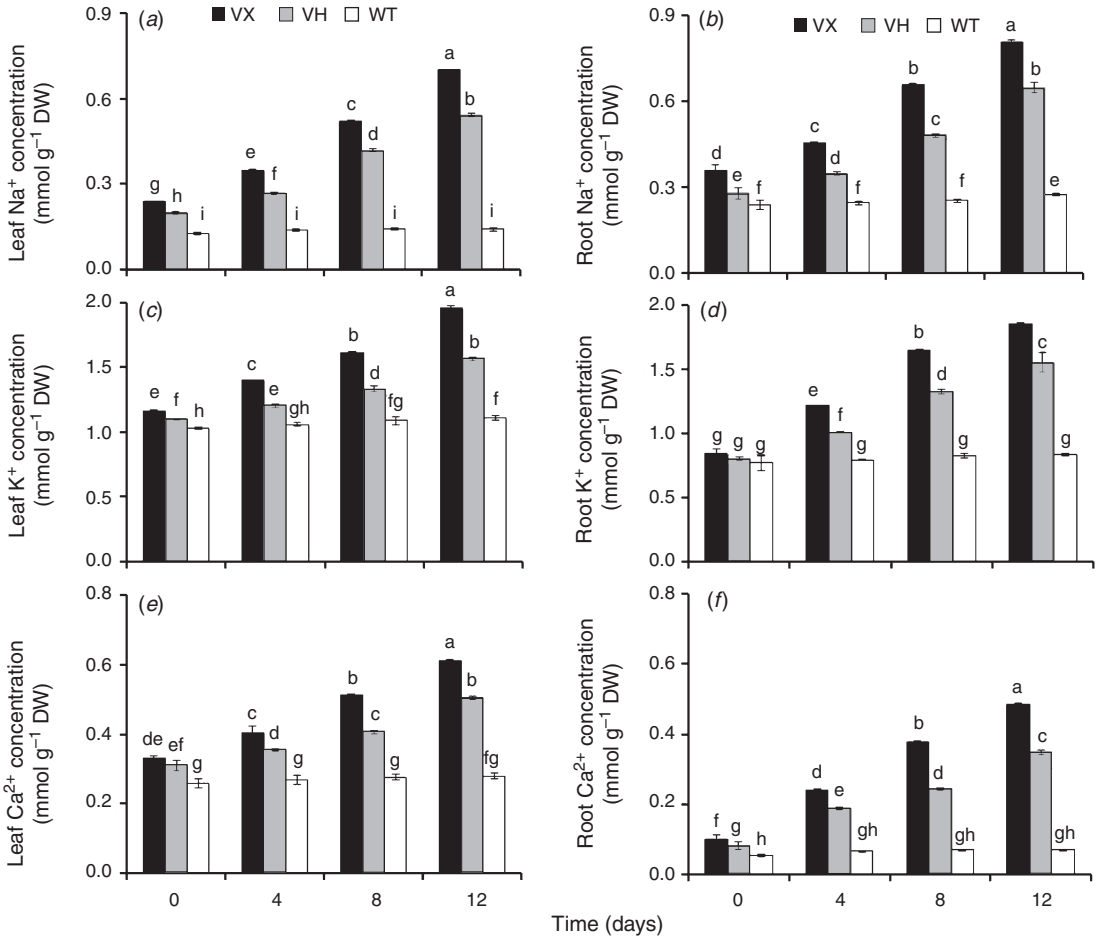

Fig. 5. Cation concentrations in leaf (left) and root (right) of transgenic and wild-type *Lotus corniculatus* during withholding water for 0, 4, 8 and 12 days respectively. Data are means ± s.e. ($n = 6$). Columns with different letters indicate significant difference at $P < 0.05$ (Duncan's test). WT, wild-type plants; VX, transgenic line co-expression *ZxNHX* and *ZxVP1-1*; VH, transgenic line expressing *ZxVP1-1*.

The enhanced salt and drought tolerance of transgenic *L. corniculatus* may be ascribed to the increased Na^+ sequestration into vacuole resulting from the expression of tonoplast Na^+/H^+ antiporter and H^+-PPase. This mechanism could provide better protection for plant cells through reducing the toxic effects of Na^+ in the cytosol, maintaining ions homeostasis especially intracellular K^+ and Na^+ homeostasis, and enhancing vacuolar osmoregulatory capacity (Gaxiola *et al.* 2007). This opinion was further supported in our study by measuring the amounts of cations: compared with WT plants, there were more Na^+, K^+ and Ca^{2+} in leaves and roots of transgenic *L. corniculatus* (Figs 4, 5). The increased accumulation of Na^+ (Figs 4*a*, *b*, 5*a*, *b*) in transgenic lines is likely caused by enhanced transport efficiency of the tonoplast Na^+/H^+ antiporter (Apse *et al.* 1999; Gaxiola *et al.* 1999) as well as increased proton motive force (Gaxiola *et al.* 2007; Bao *et al.* 2009).

We also observed that both transgenic lines exhibited better growth than WT plants even under normal conditions (Figs 2, 3), which is similar to the phenotypes of transgenic tomato (Park *et al.* 2005), cotton (Lv *et al.* 2008, 2009; Pasapula *et al.* 2011) and creeping bentgrass (Li *et al.* 2010). These are not unexpected, since several previous studies demonstrated that tonoplast H^+-PPase and Na^+/H^+ antiporter are functional in plant development. Li *et al.* (2005) found that tonoplast H^+-PPase plays an important role in controlling auxin transport and distribution, and thus regulating plant development. The expression of *Arabidopsis* tonoplast H^+-PPase stimulated shoot and root development by increased cell division at the onset of organ formation and hyperplasia in transgenic *Arabidopsis*, whereas *avp1-1* null mutants displayed severely disrupted root and shoot development (Li *et al.* 2005; Gaxiola *et al.* 2007). In contrast, a recent study showed that tonoplast H^+-PPase might regulate post germinative development in *Arabidopsis* through the hydrolysis of cytosolic PPi, which facilitates the gluconeogenesis (Ferjani *et al.* 2011). Furthermore, it was suggested that tonoplast Na^+/H^+ antiporter control cell expansion in vegetative tissues and male reproductive organs

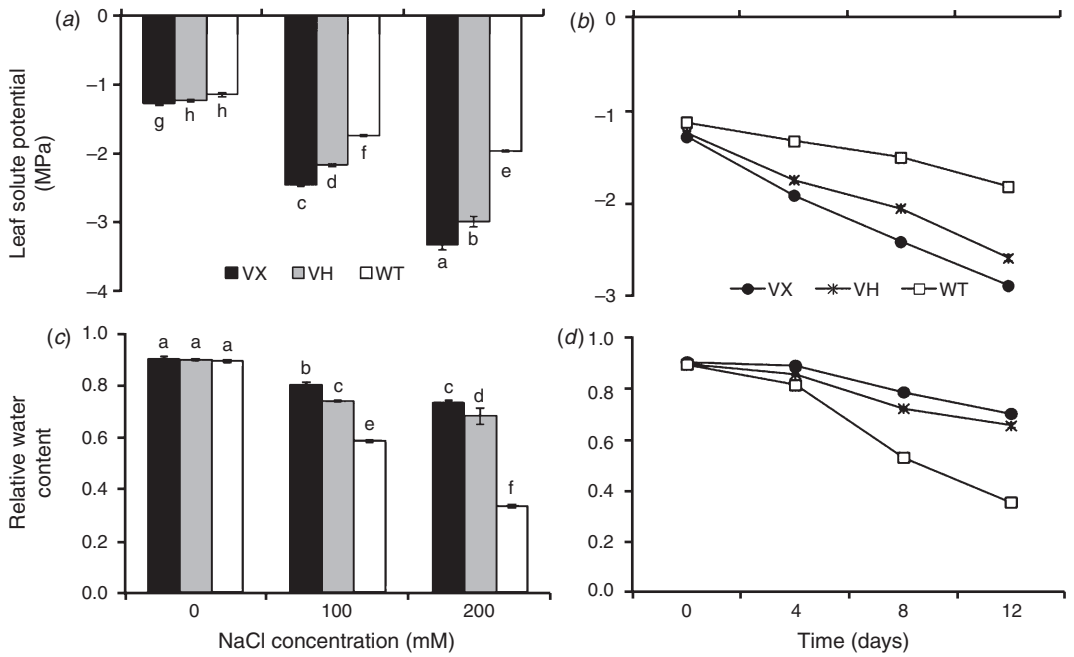

Fig. 6. Leaf solute potential (*a*, *b*) and leaf relative water content (*c*, *d*) of transgenic and wild-type *Lotus corniculatus* treated with different NaCl concentrations for 10 days (left) or withholding water for 0, 4, 8 and 12 days respectively (right). Data are means ± s.e. ($n = 6$). Columns with different letters indicate significant difference at $P < 0.05$ (Duncan's test). WT, wild-type plants; VX, transgenic line co-expression *ZxNHX* and *ZxVP1-1*; VH, transgenic line expressing *ZxVP1-1*.

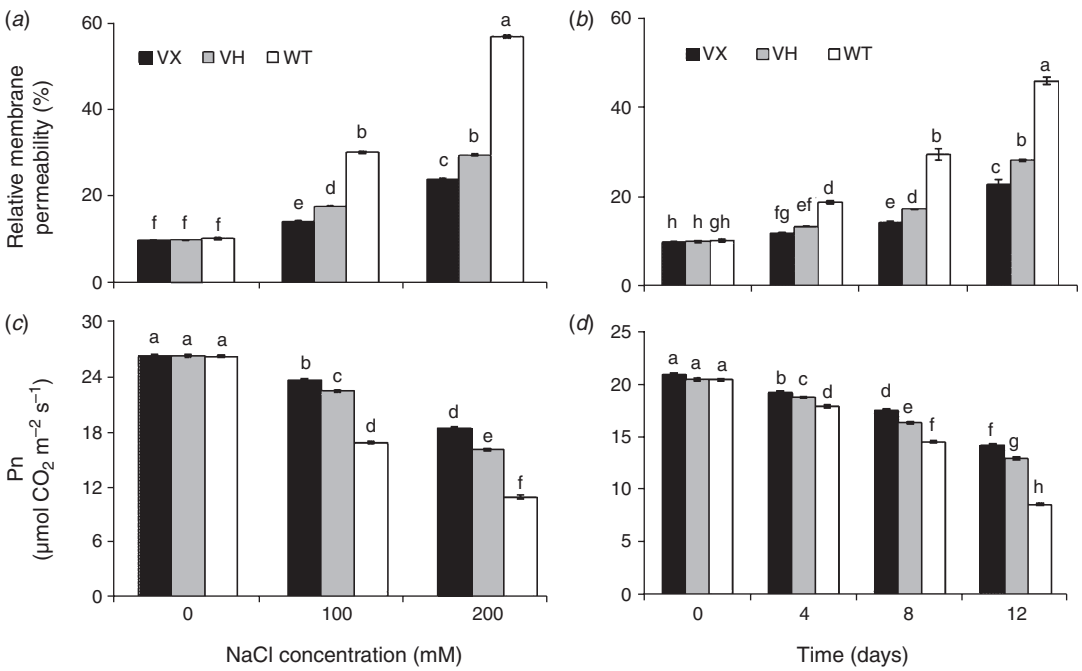

Fig. 7. Leaf relative membrane permeability (*a*, *b*) and net photosynthetic rate (*c*, *d*) of transgenic and wild-type *Lotus corniculatus* treated with different NaCl concentrations for 10 days (left) or withholding water for 0, 4, 8 and 12 days respectively (right). Data are means ± s.e. ($n = 6$). Columns with different letters indicate significant difference at $P < 0.05$ (Duncan's test). WT, wild-type plants; VX, transgenic line co-expression *ZxNHX* and *ZxVP1-1*; VH, transgenic line expressing *ZxVP1-1*.

and are required for normal flower development (Apse *et al.* 2003), which was supported by a recent evidence that *Arabidopsis* double mutant *nhx1 nhx2* plants, which were significantly smaller than the wild type (Bassil *et al.* 2011).

Expressions of ZxNHX *and* ZxVP1-1 *maintain ions homeostasis and the osmoregulation in transgenic* L. corniculatus

Controlling and re-establishing ions homeostasis, especially intracellular K^+ and Na^+ homeostasis, is of critical importance for plant adapting to salinity and water-deficit stress (Niu *et al.* 1995; Apse *et al.* 2003; Bassil *et al.* 2011). Potassium is required for plant growth, enzyme activity, cell expansion, tropisms, ion homeostasis and stomatal movements (Zhao *et al.* 2006; Bao *et al.* 2009). In most plant species, the ability to retain high cytosolic K^+ is correlative with salt and drought tolerance (Shabala 2003; Shabala and Cuin 2008). Walker *et al.* (1996) reported that the vacuolar K^+ pool is a main supplier of cytosolic K^+, because under varying external K^+ concentrations, cytosolic K^+ is tightly maintained, whereas the vacuolar K^+ pool fluctuates with K^+ supply and tissue content, implying cytosolic K^+ might be maintained partly by exchange with the vacuole. In addition to alleviating Na^+ toxicity, recent evidences demonstrated that increased vacuolar compartmentalisation can promote cellular K^+ uptake in transgenic plants, since tonoplast Na^+/H^+ antiporters also facilitate K^+ transporting into the vacuole as a K^+/H^+ exchanger in plants (Leidi *et al.* 2010; Bassil *et al.* 2011; Gouiaa *et al.* 2012). For example, the overexpression of tonoplast Na^+/H^+ antiporter led to more vacuolar K^+ accumulation and greater K^+ uptake in transgenic tomato compared with the wild type (Leidi *et al.* 2010), whereas *Arabidopsis* double mutant *nhx1 nhx2* accumulated only 30% of the wild-type K^+ concentration (Bassil *et al.* 2011). Consistent with previous studies, we found that transgenic *L. corniculatus* retained more K^+ in leaves and roots than WT plants (Figs 4*c*, *d*, 5*c*, *d*), which might be very important for transgenic plants to cope with salt and water-deficit stress. Moreover, Ca^{2+} also plays a number of roles in plant adaptation to salt and drought, such as stabilising cell walls and membrane, stimulating K^+ uptake, regulating H_2O_2 homeostasis and as a second messenger (Hirschi 2004; Conn *et al.* 2011). Our data showed that the expression of *ZxNHX* and *ZxVP1-1* significantly increased the levels of Ca^{2+} concentration in transgenic lines under salt or water-deficit treatments (Figs 4*e*, *f*, 5*e*, *f*), which may also contribute to the increased salt and drought tolerance (Bao *et al.* 2009).

Furthermore, previous studies have implied that accumulating more solutes such as K^+, Na^+ and Ca^{2+} resulting from increased compartmentalisation capacity can improve the osmoregulation capacity, which serves as a force to drive water uptake and thus, maintain the turgor of transgenic plants at low water potential media (Gaxiola *et al.* 2001; Bao *et al.* 2009; Li *et al.* 2010). Our results supported this point again. Owing to lower leaf solute potential in transgenic *L. corniculatus* (Fig. 6*a*, *b*), the leaves of transgenic plants retained more water during salt and water-deficit stress than those of WT plants (Fig. 6*c*, *d*).

Expressions of ZxNHX *and* ZxVP1-1 *protect the cell membrane and photosynthetic machinery in transgenic* L. corniculatus

It is known that salt and dehydration can cause oxidative damage to the cell membrane, and the capacity to prevent this damage is correlated with stress tolerance (Bartels and Sunkar 2005; Bao *et al.* 2009). In this study, the leaf relative membrane permeability, an indicator of cell membrane damage degree, was much lower in transgenic *L. corniculatus* than in WT under either salt or water-deficit treatments (Fig. 7*a*, *b*). Similar results were reported in many previous studies (e.g. Gao *et al.* 2006; Brini *et al.* 2007; Liu *et al.* 2010). These findings perhaps ascribed to the increased sequestration of Na^+ in the vacuole, which may protect the cells from the toxicity of excess Na^+. Furthermore, there is evidence that salt and water-deficit stress result in the reduction of photosynthetic activities, since the decrease of water content and increase of Na^+ in the cytosol can irreversibly restrain PSI and PSII (Allakhverdiev *et al.* 2000). Our data showed that the net photosynthetic rate was higher in transgenic *L. corniculatus* than in the wild-type under salt or water-deficit treatments (Fig. 7*c*, *d*). The similar phenotypes were also observed in transgenic rice co-expressing *SsNHX1* and *AVP1* (Zhao *et al.* 2006) and co-expressing *OsNHX* and *OsVP1* (Liu *et al.* 2010), and alfalfa expressing *AVP1* (Bao *et al.* 2009). These results implied that the expression of tonoplast Na^+/H^+ antiporters and H^+-PPase can protect photosynthetic machinery from Na^+ toxicity and dehydration by enhancing Na^+ compartmentalisation and other solutes accumulation in cells.

Co-expression of ZxVP1-1 *and* ZxNHX *confers even much greater salt and drought tolerance than expression of* ZxVP1-1 *alone in transgenic* L. corniculatus

Plant salt or drought tolerance is a complex trait that involves multiple physiological and biochemical mechanisms and is regulated by numerous genes (Flowers 2004; Bartels and Sunkar 2005). Although the overexpression of a single salt-responsive gene can contribute to the improvement of salt and drought tolerance to some extent, it has been observed that in many cases, the level of stress resistance is still unsuitable for the needs of practical agriculture production, even attenuate under field conditions (Singla-Pareek *et al.* 2003). Therefore, there is an urgent need to generate transgenic plants with much higher salt and drought tolerance through simultaneous expression of multiple genes. Gaxiola *et al.* (2002) proposed that simultaneous expression of both tonoplast Na^+/H^+ antiporter and H^+-pump would be required to further increase salt and drought tolerance of plants. In recent years, several groups have tested this hypothesis in rice (Zhao *et al.* 2006; Liu *et al.* 2010), tomato (Bhaskaran and Savithramma 2011) and tobacco (Gouiaa *et al.* 2012), and suggested the simultaneous expression of tonoplast Na^+/H^+ antiporter and H^+-PPase conferred greater performance to the transgenic plants than expression of the single one. Similarly, in current study, the transgenic line VX co-expressing both *ZxNHX* and *ZxVP1-1* showed a faster growth rate (Figs 2, 3), more cations accumulation (Figs 4, 5), higher leaf relative water content (Fig. 6), lower relative membrane permeability and higher net photosynthetic rate (Fig. 7), compared with the transgenic line

VH expressing single *ZxVP1-1*, under salt or water-deficit conditions. These results further demonstrate that it is a feasible way to obtain transgenic plants with even greater stress tolerance according to co-expression of tonoplast Na^+/H^+ antiporter and H^+-PPase.

In conclusion, the transgenic *L. corniculatus* lines with greater salt and drought tolerance were produced through expressing tonoplast Na^+/H^+ antiporter and H^+-PPase genes from xerophyte *Z. xanthoxylum* in this study. To our knowledge, this is the first case where the functional genes of vacuolar compartmentalisation from a xerophyte species were introduced into legume forage. It is noteworthy that co-expression of tonoplast Na^+/H^+ antiporter and H^+-PPase genes might be a more useful strategy to improve the stress tolerance of important crops.

Acknowledgements

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