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; Gouia 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 *Suueda salsa* *SsNHX1* and *Arabidopsis*
AVP1 confers higher salt tolerance than expression of the single ZsNHX1 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 TNHX1 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.

_Zygothyllium 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. (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 B5 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 transformatants 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’-GCTGGAATCAGATTTTGGA-3’) and P2 (5’-TGAGCTTATGCTGATCG-3’), whereas those for ZxNHX amplification were P3 (5’-CATTGTTTTGGTGTTC-3’) and P4 (5’-GCAGCTTACCGCCACCC-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 Alphalmager (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 398-bp fragment are P5 (5′-GGTTGCAGCCATTGTCGGT-3′) and P6 (5′-GACCTCAATCCGACACTG-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 and visualised by AlphaImager scanner (Epson America Inc., Long Beach, CA, USA). The reverse transcribed cDNA samples of 16/8 h (light/dark, the light period was a~1000 mol m⁻² s⁻¹) 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 lines and WT 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.

Salt and water-deficit treatments

To prepare the materials for physiological analysis, the T₀ 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 h/8 h (light/dark, the light flux density during the light period was 2000 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 2000 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 Ca²⁺ 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 LiNO₃ 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 Ca²⁺ 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 ρ₀ = 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. 1a). 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. 1b).

**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* (lines 1, 2, 3, 6, 8, 9, 10, 12, 13, 15), and five transgenic lines expressing *ZxVP1-1* (lines 16, 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. 2a). 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. 2b). 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. 2c). 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. 2c). 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. 2c).

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. 3a) until withholding water for 14 days when all plants wilted (Fig. 3b). 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. 3c, 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. 3e), 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.](image-url) **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.
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. 4a, 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. 4c, d). Under salt treatments, Ca2+ concentrations significantly increased in leaves and roots of two transgenic lines but did not exhibit obvious change in WT plants (Fig. 4e, 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 (Fig. 4e, f). 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. 4c, d). Under salt treatments, Ca2+ concentrations significantly increased in leaves and roots of two transgenic lines but did not exhibit obvious change in WT plants (Fig. 4e, f).

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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. 6a, b). Moreover, the leaf ψs of VX was more negative than that of VH under stress conditions (Fig. 6a, 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. 6c). 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. 6d). 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.
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. 7a, b). In transgenic lines VX and VH, the RMP was 24 and 30% under 200 mM NaCl (Fig. 7a), and was 23 and 28% after 12 days of water-deficit (Fig. 7b), 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.
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. 7c, 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. 7c); 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. 7d).

**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 sequestering 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 2005; 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.
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 4a, b, 5a, 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.
Expressions of ZsNHX and ZsVP1-1 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 correlated with salt and drought tolerance (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 and H⁺-PPase can protect photosynthetic machinery from Na⁺ toxicity and dehydration by enhancing Na⁺ compartmentalisation and other solutes accumulation in cells.

Expressions of ZsNHX and ZsVP1-1 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. 7a, 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. 7c, 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 ZsVP1-1 and ZsNHX confers even much greater salt and drought tolerance than expression of ZsVP1-1 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-resistant 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⁺-PPase 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 ZsNHX and ZsVP1-1 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
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VH expressing single ZxVP1-I, 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.

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