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Lotus tenuis **tolerates combined salinity and waterlogging: maintaining O$_2$ transport to roots and expression of an *NHX1*-like gene contribute to regulation of Na$^+$ transport**

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ABSTRACT

Salinity and waterlogging interact to reduce growth for most crop and pasture species. The combination of these stresses often cause a large increase in the rate of Na^+ and Cl^- transport to shoots; however, the mechanisms responsible for this are largely unknown. To identify mechanisms contributing to the adverse interaction between salinity and waterlogging, we compared two *Lotus* species with contrasting tolerances when grown under saline (200 mM NaCl) and O_2-deficient (stagnant) treatments. Measurements of radial O_2 loss (ROL) under stagnant conditions indicated that more O_2 reaches root tips of *L. tenuis*, compared with *L. corniculatus*. Better internal aeration would contribute to maintaining Na^+ and Cl^- transport processes in roots of *L. tenuis* exposed to stagnant-plus-NaCl treatments. *L. tenuis* root Na^+ concentrations after stagnant-plus-NaCl treatment (200 mM) were 17% higher than *L. corniculatus*, with 55% of the total plant Na^+ being accumulated in roots, compared with only 39% for *L. corniculatus*. *Lotus tenuis* accumulated more Na^+ in roots, presumably in vacuoles, thus reducing transport to the shoot (25% lower than *L. corniculatus*). A candidate gene for vacuole Na^+ accumulation, an *NHX1*-like gene, was cloned from *L. tenuis* and identity established via sequencing and yeast complementation. Transcript levels of *NHX1* in *L. tenuis* roots under stagnant-plus-NaCl treatment were the same as for aerated NaCl, whereas *L. corniculatus* roots had reduced transcript levels. Enhanced O_2 transport to roots enables regulation of Na^+ transport processes in *L. tenuis* roots, contributing to tolerance to combined salinity and waterlogging stresses.

ABBREVIATIONS

NHX, <u>Na</u>$^+$/<u>H</u>$^+$ e<u>X</u>changer; RGR, relative growth rate; ROL, radial oxygen loss

INTRODUCTION

Low O_2 concentrations often occur in saline soil due to excessive irrigation, rising water tables, poor soil structure reducing drainage, and/or seasonal flooding (Drew et al. 1988, Smedema and Shiati 2002, Rengasamy et al. 2003). Salinity and waterlogging stresses interact adversely to reduce production of crops and pastures, as very few species of agricultural relevance can tolerate the combination of salinity and waterlogging (Bennett et al. 2009). The interaction causes a large increase in the rate of transport of Na^+ and Cl^- to the shoot, in comparison with salinity alone (Drew et al. 1988, Rogers and West 1993, Galloway and Davidson 1993, Barrett-Lennard et al. 1999, Barrett-Lennard 2003). For more tolerant species, the increase in shoot Na^+ and Cl^- is less, presumably due to enhanced root aeration, for example in

certain halophytes (Colmer and Flowers 2008), *Melaleuca cuticularis* (Carter, Colmer and Veneklaas 2006), *Eucalyptus camaldulensis* (Marcar 1993), a wheat x *Thinopyrum* amphiploid (Akhtar, Gorham and Qureshi 1994), *Puccinellia ciliata* (Jenkins et al. 2010) and a perennial legume *Lotus tenuis* (Teakle, Real and Colmer 2006; Teakle et al. 2007).

Lotus tenuis Waldst. and Kit. (syn. *Lotus glaber*; Kirkbride 2006) is more tolerant to salinity and waterlogging than the more widely grown *Lotus corniculatus* (Schachtman and Kelman 1991, Rogers et al. 1997, Striker et al. 2005, Teakle et al. 2006, 2007, Real et al. 2008). Our earlier study found that for aerated saline (200 mM NaCl) treatment, there was no difference between the two *Lotus* species in xylem or shoot Na^+ concentrations and that differences in Cl^- concentrations were more correlated with tolerance to the individual stress of salinity (Teakle et al. 2006, 2007). Interestingly, despite no difference in shoot Na^+ under aerated saline treatment, shoot Na^+ was 3-fold lower in *L. tenuis* than *L. corniculatus* when salinity was combined with root-zone O_2 deficiency (Teakle et al. 2007). Tolerance to combined salinity and root-zone O_2 deficiency in *L. tenuis* was associated with lower shoot concentrations of Na^+ and Cl^- due to reduced net loading in the xylem, and higher root porosity from constitutive aerenchyma formation (Teakle et al. 2007). The aim of the present study was to identify mechanisms contributing to the adverse interaction between salinity and waterlogging on plants; thus we focused on Na^+ transport processes in this study as previous work clearly indicates that shoot Na^+ only differs between *L. tenuis* and *L. corniculatus* when salinity and waterlogging are combined, whereas Cl^- concentrations also differed under salinity alone.

Na^+ transport to the shoot depends on a number of root transport processes, including uptake and efflux of Na^+ into and from the roots, accumulation of Na^+ in root vacuoles and retrieval of Na^+ from the xylem; each of which is associated with a number of genes (Tester and Davenport 2003, Shi et al. 2002, Volkov and Amtmann 2006, Byrt et al. 2007). To identify some of the candidate genes that might be involved in Na^+ transport under combined salinity and waterlogging, in this study we focused on aspects of Na^+ transport that are most likely to be affected by root O_2 deficiency. Anoxia reduces ATP production to, at most, 37% of the ATP produced under aerated conditions and usually much less (e.g. <10%; Gibbs and Greenway 2003). Therefore, anoxic stress will cause an energy crisis for plants, unless energy demand decreases. Energy, albeit indirectly, will be required for transport processes associated with 'exclusion' of Na^+. For ion transport, energy is required for H^+-ATPase activity that maintains H^+ gradients across membranes, being essential for membrane potential and the active secondary transport of Na^+ (Greenway and Munns 1980, Barrett-Lennard 2003, Munns 2005). Potential sites of Na^+ transport in roots that rely on trans-membrane H^+ gradients, and that might therefore be impaired by O_2 deficiency, include: (1) retrieval

from the xylem, possibly via high affinity Na^+ transporters (e.g. HKT; Huang et al. 2006, James et al. 2006, Byrt et al. 2007), Na^+/H^+ antiporters (e.g. SOS1; Shi et al. 2002) or cation/H^+ exchangers (CHX; Hall et al. 2006), (2) accumulation of Na^+ in root vacuoles by tonoplast Na^+/H^+ antiporters (e.g., NHX1; Pardo et al. 2006, Xue et al. 2004), and (3) efflux of Na^+ via plasma membrane Na^+/H^+ antiporters (e.g., SOS1; Martinez-Atienza et al. 2007, Oh et al. 2007).

Experiments presented in this paper show that under combined salinity and O_2 deficiency, *L. tenuis* accumulated more Na^+ in roots than *L. corniculatus*, despite similar root:shoot ratio. This difference was presumably caused by greater accumulation of Na^+ in root vacuoles by *L. tenuis*. Subsequently, we investigated the expression of a vacuolar *NHX1*-like gene in *L. tenuis*. NHX (**N**a$^+$/**H**$^+$ e**X**changer) transporters are part of a large family of genes found in many plant species that are classified according to either a plasma membrane or subcellular (mainly vacuolar) localisation (Table 1; Pardo et al. 2006). All NHX proteins of class I (NHX1), characterised to date, are membrane transporters localised to the tonoplast. *NHX1* gene products mediate Na^+ transport into vacuoles, driven by the trans-tonoplast H^+ gradient (Horie and Schroeder 2004) established by H^+ pumps, V-ATPase and V-PPase (Gaxiola et al. 1999, Gaxiola et al. 2001, 2002; Pardo et al. 2006). Vacuolar sequestration of Na^+ protects sensitive enzymes in the cytoplasm while maintaining turgor (Greenway and Munns 1980), therefore NHX1 transporters play a role in salt tolerance (Apse et al. 1999, Zhang and Blumwald 2001). ATP-limited conditions, caused by respiration being inhibited by O_2 deficiency in roots, could affect the function of NHX1 transporters and hence the accumulation of Na^+ into vacuoles under high NaCl concentrations.

Despite several studies on the individual stresses of salinity and waterlogging (and hypoxia or anoxia), very little research has been undertaken on the combination of these stresses, which can interact to affect plants more severely than the additive effect of each stress independently (Barrett-Lennard 2003). Little is known about the physiological basis of tolerance to combined salinity and waterlogging and what genes might be involved. We compared the responses to combined salinity and root-zone O_2 deficiency in two closely related *Lotus* species that have previously been found differ in tolerance to combined salinity and waterlogging (Teakle et al. 2006, 2007). The objective of the present study was to assess root O_2 transport and consumption, as well as responses of tissue ions to combined salinity and root-zone O_2 deficiency. In addition, we isolated and functionally characterised an *NHX1*-like gene from *L. tenuis* to determine if the combination of salinity and root-zone O_2 deficiency affected expression of a gene that is likely to be associated with accumulation of Na^+ in root vacuoles.

MATERIALS AND METHODS

Plant growth and ion concentrations in response to NaCl and stagnant root-zone treatments

Scarified seeds of *Lotus tenuis* (cv. Chaja) and *L. corniculatus* (cv. San Gabriel) were washed briefly with 0.04% (w/v) bleach (NaHClO) and then thoroughly rinsed with de-ionized (DI) water. Seeds were imbibed for 3 h in aerated 0.5 mM $CaSO_4$ in darkness then placed on a mesh screen over aerated 10% concentration nutrient solution and kept in darkness for 3 d. The full strength nutrient solution consisted of macronutrients (mM): 0.5 KH_2PO_4, 3.0 KNO_3, 4.0 $Ca(NO_3)_2$, 1.0 $MgSO_4$; and micronutrients (µM): 37.5 $FeNa_3EDTA$, 23.0 H_3BO_3, 4.5 $MnCl_2$, 4.0 $ZnSO_4$, 1.5 $CuSO_4$ and 0.05 MoO_3. Solution pH was buffered with 2.5 mM MES (2-[N-Morpholino] ethanesulfonic acid) adjusted with KOH to 6.3. After 3 d, seedlings were transferred to 25% nutrient solution still on mesh and exposed to light. After a further 4 d, individual seedlings were transplanted to 50% nutrient solution and another 7 d later the solution was changed to 100% concentration. Nutrient solutions were renewed weekly and topped up with DI water as required. Each treatment x species combination was represented by four replicate pots (4 L capacity) in a completely randomised block design where blocks were designated according to position in a phytotron (20/15˚C day/night). Average photosynthetically active radiation (PAR) in the phytotron at midday during the experimental period was 1105µmol m^{-2} s^{-1}.

Four treatments were imposed 28 d after imbibition and these were: an aerated control (0.1 mM NaCl); a saline treatment (aerated, 200 mM NaCl); a stagnant non-saline treatment (non-aerated, 0.1 mM NaCl) and the combination of stagnant-plus-saline (non-aerated, 200 mM NaCl). NaCl was added in daily 50 mM increments until the final concentration of 200 mM. Hypoxia was imposed 24 h later in all pots assigned to stagnant and stagnant-plus-saline treatments, by bubbling with N_2 gas until the dissolved O_2 level was less than approximately 10% of air-saturated solution, so as to give a hypoxic pre-treatment and thus avoid subsequent 'anoxic shock' (Gibbs and Greenway 2003). The following day, the nutrient solution in these pots was changed to a stagnant deoxygenated (i.e. anoxic) 0.1% (w/v) agar solution. This stagnant treatment simulates the decrease in dissolved O_2 and increase in ethylene that occurs under waterlogged conditions to provide a root-zone O_2 deficiency treatment (Wiengweera et al.1997).

Plants were harvested 0, 7 and 14 d after saline treatments commenced. Four plants per replicate were combined for measurements to minimize the influence of plant-to-plant variability in these cross-pollinated and thus variable species. Results are expressed on a per plant basis. For all harvests, roots were rinsed 3 times, for 10 s each time, in 4.0 mM $CaSO_4$ plus mannitol at iso-osmotic concentrations to the

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saline treatment. Osmotic coefficients for NaCl were taken from Lang (1967). Shoots were separated from roots and stem bases gently rinsed in DI water and blotted dry. Sub-samples of leaf and root tissue were frozen in liquid nitrogen and stored at -80°C for RNA extractions (see below). The remaining root and shoot tissues had fresh weights recorded and were oven-dried for 3 d at 70°C. Dry weights were recorded and total dry weight calculated including the RNA tissue sub-samples based on fresh:dry weight ratios. Relative growth rate (RGR) was calculated from the natural log of the difference in dry weights between harvests taken 7 and 14 d after treatments commenced (estimator 2; Hoffman and Porter 2002). Na^+, K^+ and Cl^- concentrations were measured in dried root and shoot samples that had been ground to a fine powder. Approximately 100 mg of tissue (exact weight recorded) was extracted with 10 ml of 0.5 M HNO_3 and incubated with gentle shaking for 48 h in darkness at 30°C. Diluted extracts were analysed for Na^+, K^+ (Jenway PFP7 flame photometer, Essex, UK) and Cl^- (Buchler-Cotlove Chloridometer 662201, New Jersey, USA). The reliability of these analyses was confirmed by taking a reference plant sample (ASPAC #85) with known ionic composition through the same procedures. Rates of net transport of Na^+ between 7 and 14 d treatment were calculated following the method of Williams (1948).

Root radial O_2 loss and O_2 consumption rates

An extra plant per replicate was harvested from the above experiment after 14 d stagnant treatment to measure radial O_2 loss (ROL) from intact lateral roots. ROL was measured in a 20°C controlled temperature room using root-sleeving O_2 electrodes (Armstrong and Wright 1975, Armstrong 1979). The shoot of each plant was in air and the root/shoot junction was sealed in a rubber lid with wet cotton wool. The intact root system was immersed in a clear Perspex chamber (50 mm x 50 mm x 150 mm, w x b x h) containing O_2-free stagnant solution with 0.1% agar (w/v), 0.5 mM $CaSO_4$, 5.0 mM KCl. The plants were left for approximately 1 h to equilibrate before measurements started. ROL was measured every 5 to 10 mm along intact lateral roots (~70 to 90 mm in length); these emerged from just below the root/shoot junction and had no further branching laterals. One intact root was measured for each of four plants, providing four replicates. The diameter of the studied root was determined using an Axiovert 100 microscope (Carl Zeiss, Jena, Germany). Stagnant-plus-saline roots of *L. corniculatus* were too small to be measured using this method, therefore this treatment is not included.

O_2 consumption rates by excised root tissues were measured polarographically according to Lambers and Steingröver (1978). Approximately 2 cm root tips were cut with a sharp scalpel from lateral roots (~1 g total root tips) and were added to a 50 ml cuvette with a magnetic stirrer containing a fresh solution of the same composition as the aerated nutrient solution plants were grown in, i.e. with or without 200 mM

NaCl. The The rate of O_2 depletion was measured at 20°C immediately after roots were excised. A linear depletion of O_2 was measured for at least 10 min. Roots from four different plants per treatment and species were measured separately, giving four replicates. These measurements were taken in air-saturated solutions, so root tips from stagnant treatments were not included as possible effects from re-exposure to air were of concern.

Cloning of an *NHX1*-like gene in *Lotus tenuis*

Due to a lack of sequence information for *L. tenuis*, primers were designed based on homology to other legumes with *NHX1*-like genes sequenced. Using BLASTn (Altschul et al. 1997), conserved regions of *Glycine max* (AY392759), *Medicago sativa* (AY456096) and *Trifolium repens* (EU109427) *NHX1*-like genes were identified. The conserved sequences were aligned to the publicly available *Lotus japonicus* genome and primers were then designed based on *L. japonicus* nucleotide sequences, which were more likely to be similar to *L. tenuis*. Primers LjF and LjR (Table 1) successfully amplified *L. tenuis* cDNA using GoTaq® DNA Polymerase (Promega). All PCRs were done with an initial denaturation of 2 min at 94°C, followed by 30 cycles of: 94°C for 30 s, anneal 50-60°C for 30 s, extension at 72°C for 0.5-3 min; and a final extension at 72°C for 10 min and hold at 4°C. Specific annealing temperatures and extension times for each primer pair are given in Table 1. PCR products were visualized on ethidium bromide stained 1% agarose gels using electrophoresis. The PCR product from LjF and LjR was purified using Wizard® SV Gel Clean-up System (Promega) and used as a probe to screen a *L. tenuis* cDNA library for full-length *NHX1*-like genes.

The *L. tenuis* cDNA library was constructed using root tissue of plants exposed to 200 mM NaCl for 24 h. Roots were finely ground using a Retsch tissue lyser (Qiagen, Hahn, Germany) at 25 Hz for 1 min. Total RNA was extracted using RNeasy® Mini Kit (Qiagen) and converted to mRNA using the Oligotex® mRNA kit (Qiagen). The cDNA library was constructed from 1 µg of mRNA in a Gateway™ vector system using the CloneMiner™ cDNA Library Construction kit (Invitrogen). The cDNA library size was estimated to contain approximately 10^7 colony forming units (cfu). Twenty-five colonies were selected at random and inserts sequenced. Average insert size was 1.9 kb and ranged from 0.2 to 4 kb with 96% recombination, demonstrating a good quality cDNA library with potential to obtain full-length genes.

The cDNA library was spread onto 15 cm diameter LB kanamycin agar plates at approximately 30,000 clones per plate and incubated overnight at 37°C. Nytran® N filters (Whatman) were used for colony lifts. The purified PCR product from primers LjF and LjR (Table 1) was labelled with Redivue ^{32}P-dCTP using

Rediprime™ II DNA Labelling System and purified using Nick™ columns (Amersham). The filters with fixed colonies were incubated with the probe in hybridisation bottles overnight at 65°C on a turning rack. Hybridized filters were washed stringently at 60°C in 2 x SSC, 0.1% SDS; 1 x SSC, 0.1% SDS; 0.5 x SSC, 0.1% SDS; 0.1 x SSC, 0.1% SDS. Filters were sealed in Saran wrap and exposed to X-ray film (Kodak MxB) at -70°C. After a minimum of 3 d, the film was developed in a X-umat (Xograph Compact X4, Tetbury, UK). Positive colonies were streaked onto new plates and re-screened as above. Colonies containing an NHX1-like insert were confirmed by PCR and sequencing of plasmid DNA (extracted using QiaPrep® Minprep kit from Qiagen). Sequencing was done using BigDye™ terminator cycling conditions. The reacted products were purified by ethanol precipitation and sequenced using an ABI3730XL automatic sequencer (Macrogen Inc).

Sequences of *L. tenuis* plasmid DNA isolated from positive library clones were compared to known *NHX* genes using BLAST. The amino acid sequence of full-length coding regions was predicted using ExPASy DNA translation programs (Gasteiger et al. 2003). Sequences were aligned using CLUSTALW (Thompson, Higgins and Gibson 1994) and protein structure estimated from a consensus of nine different programs using ConPred II (Arai et al. 2004). Subcellular localisation was predicted using WoLF PSORT (Horton et al. 2006).

Functional expression of *Lotus tenuis NHX1*-like gene in yeast

The *L. tenuis NHX1*-like gene was sub-cloned into pYES-DEST52 yeast expression vector by LR recombination (see Gateway™ Teachnology Manual, Invitrogen) and confirmed by sequencing from the 5´end. This vector construct (*LtNHX1*) and the empty pYES-DEST52 vector alone (empty vector, *EV*) were used to transform the yeast strain AXT3 (*MATα, Δena1::HIS3::ena4, Δnha1::LEU2, nhx1::TRP1, ura3-1, ade 2-1, can1-100*). This strain lacks the endogenous yeast NHX1 protein and the plasma membrane Na^+ efflux transporters ENA1-4 and NHA1 (Quintero et al. 2000). AXT3 is derived from W303 (*MATα, ura3-1, ade 2-1, can1-100, leu2-13, ade2-1, his3-11,15*) obtained from the Yeast Genetic Resource Center (strain BY20135, Osaka City University, Japan), and this generic strain was used as the wild type control (*WT*) in all experiments. Yeast transformations were based on a modified LiAc protocol by Gietz et al. (1992) and transformants isolated on synthetic complete (SC) medium supplemented with all amino acids and nutrients (Sherman 1991), except uracil. Yeasts were maintained on YPD plates (1% yeast extract, 2% peptone, 2% glucose, 2% agar).

Single colonies of *WT*, AXT3 x *EV* and AXT3 x *LtNHX1* were inoculated in 5.0 ml SC minus uracil (plus uracil for *WT*) media and incubated at 30°C overnight. YPGAL (1% yeast extract, 2% peptone, 2% galactose) was added to overnight cultures (to induce protein expression) and cultures were incubated until OD_{600} of 0.6 was obtained (about 2-4 h). Cultures were serially diluted from 10^{-1} to 10^{-3} and 5 µl of each dilution was pipetted onto YPGAL plates (2% agar) with 0, 50, 75 and 100 mM NaCl. Plates were incubated at 30°C for 4 d and then photographed. Experiments were repeated with three individual colonies from two independent transformations and similar results obtained. For measuring intracellular ion concentrations, overnight inoculations were re-suspended in YPGAL plus 100 mM NaCl. Cultures of 5 ml were taken after 0, 2, 4, 6 and 8 h NaCl treatment. Samples were centrifuged for 5 min at 2000 *g*, washed twice in iso-osmotic sorbitol with 20 mM $MgSO_4$ and then extracted overnight in 10 ml of 0.5 M HNO_3. Na^+, K^+ and Cl^- were measured in extracts as described above.

Quantitative real-time PCR

RNA was extracted from leaf and root tissues harvested from all four treatments (see above) for both *L. tenuis* and *L. corniculatus*. Liquid N_2 frozen samples were finely ground in a tissue lyser and RNA extracted based on the guanidine thiocyanate protocol of Chomczynski (1993). Quality and quantity of RNA was checked using a biophotometer (Eppendorf AG, Hamburg, Germany) and integrity checked by electrophoresis on 1% agarose gels. Any genomic DNA was removed using RQ1 RNase-free DNase (Promega). First strand synthesis of cDNA from 1 ug RNA was done with Oligo(dT)$_{18}$ primer using M-MLV reverse transcriptase (RNase H minus point mutant, Promega) and incubated at 40°C for 10 min, 48°C for 50 min and 70°C for 15 min.

For qPCR, primers NQF and NQR (Table 1) were designed at the 3´ end of the *L. tenuis NHX1*-like gene (*LtNHX1*) cloned from the cDNA library. Reported 'housekeeping' genes homologous to actin (*ACT2*), elongation factor *α* (*EF-1α*) and β-tubulin (*TUB*) were cloned and partially sequenced from the *L. tenuis* cDNA library as above. The most stable gene expression across all treatments in this experiment for both *L. tenuis* and *L. corniculatus* was β-tubulin, so this 'housekeeping' gene was used as an internal reference for qPCR (primers TQF and TQR, Table 1). The 3´ end of the *L. tenuis TUB1* (*LtTUB1*) gene where primers TQF and TQR were designed was also conserved with a *L. corniculatus* β-tubulin gene (Accession number #AY633708); making *LtTUB1* a preferred choice of 'housekeeping' gene for these species. Standards for qPCR were made for each gene using purified PCR products of *LtTUB1* and *LtNHX1* that were serially diluted from 5 x 10^{-1} to 5 x 10^{-7} pg DNA.

Reactions for qPCR (15 µl) consisted of: 2 x SybrGreen master mix (iQ^{TM} Supermix), 0.3 µM primers, and 20 ng template cDNA. Standards, cDNA (3 biological replicates for each treatment x species combination) and negative controls were run in duplicate on 96 well plates in a Biorad iCycler iQ^{TM} Real-time PCR Detection System. The protocol used was: initial denaturation of 95°C for 2 min, 40 cycles of 95°C for 30 s, 52°C for 30 s (real time data collected at this point) and 72°C for 30 s; final extension at 72°C for 7 min. Melt curve analysis (starting at 70°C for 50 cycles of 0.5°C increment) confirmed the specificity of the primers. PCR efficiency was calculated and all standard curves had an R^2 of 0.99 or higher. Each run was repeated twice to give four technical replicates for each biological replicate. Results were analysed using Qbase software (Hellemans et al. 2007), with *LtNHX1* data normalised to *LtTUB1*.

Data analyses

All statistical analyses used Genstat for Windows 10th Edition (Genstat software, VSN International, Hemel Hempstead). Residuals were checked for normality and homogeneity and no transformations were necessary. ANOVA was used to check for overall significant differences and interactions between species and treatments. The treatment means were tested for significant differences between species using Tukey's test or paired t-tests, depending on the complexity of the data set. Unless otherwise stated, significance level was $P \leq 0.05$. Graphs of means and standard errors (SE) were made using SigmaPlot for Windows (version 10.0).

RESULTS

***Lotus tenuis* exhibits a 'partial' barrier to radial O$_2$ loss**

Rates of radial O$_2$ loss (ROL) were measured along lateral roots of stagnant-treated *L. tenuis* and *L. corniculatus*. *L. tenuis* exhibited a 'partial' barrier to ROL (see Colmer 2003), as the O$_2$ flux from the lateral roots did not differ significantly for 10 to 50 mm from the root tip (Fig. 1). At distances of 30 mm or less from the root tip, *L. tenuis* had twice the O$_2$ flux of *L. corniculatus* roots. The stagnant-plus-saline treatment reduced the O$_2$ flux from *L. tenuis* root tips by half (data not shown) but could not be measured for *L. corniculatus* roots from this treatment owing to the small roots formed. The higher ROL near root tips of *L. tenuis*, compared with *L. corniculatus*, would have been due to better internal O$_2$ movement resulting from higher root porosity (Teakle et al. 2007) and the partial barrier to ROL, but was not due to differences in respiratory demand for O$_2$ between root tissues of *L. tenuis* and *L. corniculatus* (Fig. 2).

***Lotus tenuis* restricts root to shoot transport of Na^{+} under combined saline and stagnant root-zone treatments**

Concentrations of Cl^{-}, Na^{+} and K^{+} were measured in roots and shoots of *L. tenuis* and *L. corniculatus* in saline and stagnant root-zone treatments. Concentrations of all three ions measured for aerated controls and stagnant non-saline (0.1 mM NaCl) treatments after 7 and 14 d, did not differ significantly from concentrations measured at the time treatments were imposed (i.e. at 0 d, Fig. 3), and also did not differ significantly between *L. tenuis* and *L. corniculatus* for roots or for shoots (data not shown). Therefore, we only present results for the saline and stagnant-plus-saline treatments, in which the ion concentrations did significantly increase (Na^{+} and Cl^{-}) or decrease (K^{+}) during the treatment period for both species.

After 7 and 14 d saline (200 mM NaCl) treatment, *L. corniculatus* had 1.6 and 1.9-fold higher shoot Cl^{-} concentrations, respectively, compared with *L. tenuis* (Fig. 3a). Root Cl^{-} concentrations did not differ between the two species for up to 14 d saline treatment. Compared with aerated saline treatment, the stagnant-plus-saline treatment caused shoot Cl^{-} to increase from 1550 to 2365 µmol g^{-1} DW for *L. corniculatus* and from 812 to 1443 µmol g^{-1} DW for *L. tenuis* after 14 d (Fig. 3b). Interestingly, despite *L. tenuis* having lower shoot Cl^{-} than *L. corniculatus*, root Cl^{-} concentration of *L. tenuis* was 32% higher than *L. corniculatus* after 14 d stagnant-plus-saline treatment. By contrast with Cl^{-}, there was no significant difference between the two *Lotus* species in shoot Na^{+} after 7 or 14 d aerated saline treatment (Fig. 3c). However, large differences in shoot Na^{+} between the species were observed in the stagnant-plus-saline treatment. In comparison with the aerated saline treatment, shoot Na^{+} increased by 1.5-fold to 2670 µmol g^{-1} DW for *L. corniculatus* and by 1.2-fold to 2010 µmol g^{-1} DW for *L. tenuis* after 14 d (Fig. 3d). Thus, *L. corniculatus* had 25% higher shoot Na^{+} than *L. tenuis* under stagnant-plus-saline treatment. Despite the lower shoot Na^{+} concentration of *L. tenuis* compared with *L. corniculatus*, under the combined treatment *L. tenuis* had 17% higher root Na^{+} concentration (Fig. 3c and 3d).

To determine the significance of the difference in root Na^{+} concentration between the two *Lotus* species, we calculated net rates of Na^{+} transport to the shoot and Na^{+} contents of the whole plant and root system (Table 2). *Lotus tenuis* had a lower net rate of transport of Na^{+} to the shoot in the stagnant-plus-saline treatment, compared with *L. corniculatus* (607 versus 786 µmol g^{-1} root DW d[-1]). In addition, under stagnant-plus-saline treatment, the proportion of Na^{+} in the roots decreased to 39% for *L. corniculatus* (compared with 57% for saline treatment), while in the roots of *L. tenuis* it was maintained at 55% of the total Na^{+} (not significantly different from the aerated saline treatment; Table 2). The significance of this result is highlighted by both species having similar total plant Na^{+} content in the stagnant-plus-saline

treatment. For example, *L. tenuis* retains 55% of total plant Na^+ in roots, giving 457 and 375 μmols in roots and in shoots respectively. By contrast, *L. corniculatus* only retains 39% in roots, giving 352 and 551 μmols in roots and shoots, respectively. This mass balance shows that differences in root retention of Na^+ are contributing significantly to differences between the two species in shoot Na^+ concentrations for the stagnant-plus-saline treatment. Furthermore, there was no significant difference between the two species in root:shoot mass ratio (data not shown) within any of the treatments. Taken together, these results suggest that in the stagnant-plus-saline treatment, *L. tenuis* is able to retain a higher proportion of Na^+ in roots compared with *L. corniculatus* (55 versus 39%) and has a lower net rate of Na^+ transport to the shoot, therefore having 25% lower shoot Na^+ concentration than *L. corniculatus*.

After 14 d of saline or stagnant-plus-saline treatments, there were no significant differences between the two species in shoot or root K^+ concentrations. Concentrations of K^+ in roots and shoots had declined at 14 d for both *L. tenuis* and *L. corniculatus* after the addition of saline or stagnant-plus-saline treatments to about 1000 and 530 μmol g^{-1} DW, respectively (Fig. 3e and 3f). Root K^+ concentrations decreased by less than shoots, with concentrations 35% higher than in shoots for saline treatment and 54% higher for stagnant-plus-saline treatment.

We measured relative growth rates (RGR) between 7 and 14 d of treatments. Root and shoot RGR were similar, thus total plant RGR is presented (Fig. 4). RGR for stagnant-treated plants did not differ significantly from controls in this time period, indicating the high waterlogging tolerance of both species. RGR for saline treated plants was 63% of control for both species. For *L. tenuis,* RGR for stagnant-plus-saline was the same as for the aerated saline treatment. By contrast, the RGR of *L. corniculatus* for stagnant-plus-saline decreased to only 44% of control (0.073 g g^{-1} d^{-1}), which was also 30% lower than *L. tenuis* (0.106 g g^{-1} d^{-1}). In summary, RGR only differed between the two species in the stagnant-plus-saline treatment, when *L. corniculatus* had 25% higher shoot Na^+ and 39% higher shoot Cl^- concentrations, which likely would have contributed to the lower RGR.

Sequence analysis of a *Lotus tenuis NHX1*-like gene

Sequencing results and BLAST analysis confirmed several of the positive colonies from our *L. tenuis* cDNA library contained inserts with the full coding region of an *NHX1*-like gene. Each part of the full-length gene was independently sequenced (from individual plasmids) a minimum of three times. The predicted open reading frame (ORF) was 1632 bp (Genbank accession # EU727217), encoding a protein

of 543 amino acids (Fig. 6). The protein is predicted to have 11 transmembrane spanning domains localised to the tonoplast (Fig. 5a), similar to other NHX genes that all have 10-12 transmembrane regions (Pardo et al. 2006). When compared to the six characterised *Arabidopsis thaliana* NHX genes, the *L. tenuis* gene was most similar at the nucleotide level to *AtNHX1* (Fig. 5b), therefore we will refer to the *L. tenuis* gene as *LtNHX1*. The LtNHX1 protein structure is predicted to have a hydrophilic C-terminal in the cytoplasm, with the N-terminus in the vacuole, as per AtNHX1 and human NHE1 (Sato and Sakaguchi 2005). A multiple alignment analysis indicates high similarity between LtNHX1 and other known plant NHX1-like proteins (Fig. 5c and Fig. 6), in particular to other legumes such as *Glycine max* and *Trifolium repens* with 88.5% and 85.5% similarities at the protein level, respectively. A completely conserved amiloride-binding domain (^{84}LFFIYLLPPI^{93}) was found in the third transmembrane region (Fig. 6).

LtNHX1 **improves salt tolerance of a yeast mutant**

The yeast strain AXT3, lacking all endogenous Na^+ transporters, was transformed with *LtNHX1*. In comparison with wild type yeast, AXT3 is highly salt sensitive, with growth already reduced at 25 mM NaCl (data not shown), and no growth evident at 100 mM NaCl (Fig. 7a). The expression of *LtNHX1* improved the salt tolerance of AXT3, with substantial yeast growth now clearly visible at 100 mM NaCl. After 8 h of 100 mM NaCl treatment, the intracellular concentration of Na^+ in *LtNHX1*xAXT3 was 357 nmol mg^{-1} DW, being only 10% less than in AXT3, and the K^+ concentration did not differ between the two strains (Fig. 7b). Cl^- concentrations in AXT3 transformed with *LtNHX* or empty vector were not significantly different to that in the wild type and were all less than 20 nmol mg^{-1} DW (data not shown). Therefore, the addition of *LtNHX1* to AXT3 most likely improved the salt tolerance of the yeast mutant by greater accumulation of Na^+ in vacuoles.

Expression of *NHX1* in *Lotus tenuis* and *L. corniculatus*

The expression of *NHX1* was studied using quantitative real-time PCR (qPCR). RNA was extracted from young leaves and roots of saline and stagnant treated *L. tenuis* and *L. corniculatus*. High quality RNA extracts were obtained, with one exception. Despite employing a range of methods to extract RNA, only poor quality RNA was obtained from stagnant-treated *L. corniculatus*, presumably due to an unknown compound that forms in response to this growth condition inhibiting the extraction procedure. Therefore, data for this treatment are not available for *L. corniculatus* and thus not presented. In both species, transcript levels of *NHX1* in roots increased by 2 to 3-fold in response to salinity (Fig. 8). In *L. tenuis* plants under stagnant-plus-saline conditions, root transcript levels of *NHX1* were similar to aerated saline-

treated plants (up-regulated by 2-fold compared with control). By contrast, in *L. corniculatus* transcript levels were about 3-fold lower in stagnant-plus-saline than in aerated-saline treatment, and hence similar to levels in control roots. Thus, under stagnant-plus-saline treatment, transcript levels of *NHX1* in roots of *L. tenuis* were about 4-fold higher than in *L. corniculatus*.

DISCUSSION

Saline lands are often also subject to transient waterlogging (Ghassemi et al. 1995); therefore tolerance to these stresses combined can be essential for agricultural plants to be productive in these areas (Bennett, Barrett-Lennard and Colmer 2009). The interaction of waterlogging with salinity causes a large increase in the rate of transport of Na^+ and Cl^- to the shoot, in comparison to salinity alone (Barrett-Lennard 2003), and is most likely caused by decreased 'exclusion' of these ions from the xylem (Teakle et al. 2007). Using two *Lotus* species that differ in tolerance to stagnant-plus-saline conditions, the present study showed that stagnant-plus-saline stress causes a fast (within 24 h) increase in shoot Na^+ and Cl^- for the more sensitive species, *L. corniculatus*, also reducing RGR by 30% compared with *L. tenuis*. The main difference between aerated saline treatment and the stagnant saline treatment was the higher accumulation of Na^+ in shoots of *L. corniculatus* compared with *L. tenuis.* Tolerance to stagnant-plus-saline treatment in *L. tenuis* was also associated with the accumulation of a larger proportion of Na^+ in roots, most likely sequestered in root vacuoles. Better root aeration due to higher root porosity (Teakle et al. 2007) and a 'partial' barrier to ROL (present study) presumably enables continued functioning of ion transport processes in roots of *L. tenuis*, whereas O_2 supply to roots of *L. corniculatus* was much less in stagnant conditions (Fig. 1). Enhanced root O_2 supply would help maintain expression of an *NHX1*-like gene in *L. tenuis* roots and thus may have contributed to the vacuolar sequestration of Na^+ in roots to reduce shoot uptake.

Waterlogging reduces O_2 availability in roots, thus mechanisms to enhance root aeration develop in waterlogging-tolerant species (Colmer and Voesenek 2009, Licausi and Perata 2009). These mechanisms include extensive development of adventitious roots (Vartapetian and Jackson 1997), aerenchyma formation (Armstrong 1979), a barrier to ROL (Armstrong 1979, Colmer 2003) and the induction of anaerobic metabolism in anoxic tissues (Gibbs and Greenway 2003). Tolerance to waterlogging in *L. tenuis* was previously found to be associated with high (15-35%) root porosity due to constitutive development of aerenchyma in lateral roots (Teakle et al. 2006, 2007). The present study showed that *L. tenuis* also exhibits a partial barrier to ROL, which combined with aerenchyma in root tissues, would

enable more O_2 to reach root tips (Fig. 1). The O_2 flux at tips of roots in anoxia, relying on internal transport via aerenchyma), was for *Lotus tenuis* twice that of *L. corniculatus*, which likely impacts on cellular processes in roots, in particular energy-dependant ion transport. O_2 deficiency reduces ATP production by at least 60% (Gibbs and Greenway 2003), which will impair H^+-ATPase activity and thus diminish trans-membrane H^+ gradients essential for many ion transporters, and is the likely cause of increased shoot Na^+ and Cl^- during combined waterlogging and saline conditions (Barrett-Lennard 2003). For example, in maize roots without aerenchyma, O_2 deficiency caused an immediate flux of Na^+ to shoots, even at lower NaCl concentrations (1- 50 mM; Drew et al. 1988). Similarly, *Trifolium* species with extensive adventitious root development, with aerenchyma occupying 14% of the root volume, were better able to 'exclude' Na^+ and Cl^- from shoots after 15 d flooding combined with 60 mM NaCl treatment (Rogers and West 1993). These results, similar to our present findings of better root aeration in *L. tenuis*, demonstrate the importance of enhanced root aeration for maintaining ion transport processes in roots to prevent large increases in shoot Na^+ and Cl^-.

Restricting root-to-shoot transport of Na^+ and Cl^- is critical for tolerance to salinity and waterlogging stresses combined (Barrett-Lennard 2003, Colmer and Flowers 2008). Interestingly, *L. corniculatus* had 25% higher shoot Na^+ than *L. tenuis* in stagnant-plus-saline treatment, despite there being no significant difference between the two species for the aerated saline treatment. This result suggested that a breakdown in Na^+ transport processes was a key factor contributing to the sensitivity of *L. corniculatus* to combined salinity and waterlogging, as differences in Cl^- concentrations between the species were consistent between the aerated-saline and stagnant-saline treatments. *L. tenuis* was able to retain a higher proportion of the total Na^+ in roots compared with *L. corniculatus* in the stagnant saline treatment (55% versus 39%, Table 1). Furthermore, after 9 d stagnant-plus-saline treatment, *L. tenuis* had 17% higher root Na^+ concentrations than *L. corniculatus* (Fig. 3), despite similar total plant Na^+ content (Table 1). Taken together, the results indicate that *L. tenuis* has a lower rate of Na^+ transport to shoots as it accumulates a larger proportion of the total plant Na^+ in roots. The distribution of Na^+ between roots and shoots contributes to salt tolerance. For example, tolerant wheat genotypes had lower shoot Na^+, but higher root Na^+ concentrations, suggesting more retention of Na^+ in roots compared with sensitive genotypes (Husain et al. 2004). Na^+ retained in roots is presumably being accumulated in vacuoles, as cytosolic enzymes are sensitive to Na^+ in all species (Flowers et al. 1977).

The accumulation of Na^+ in vacuoles relies on energy-dependent transport processes that could be affected by O_2 deficiency in roots. Accumulation of Na^+ in vacuoles occurs via Na^+/H^+ antiport driven by the H^+ difference across the tonoplast, which relies on the activity of H^+ pumps V-ATPase and V-PPase (Gaxiola

et al. 2001, 2002, Pardo et al. 2006). Although V-PPase activity might partially compensate for reduced V-ATPase activity (Huang et al. 2005), a diminished trans-tonoplast H^+ difference under ATP limiting conditions caused by O_2 deficiency could impair antiport activity. We cloned an *NHX1*-like gene from *L. tenuis* (*LtNHX1*, Genbank accession # EU727217) to determine the effect of stagnant-plus-saline treatment on the expression of a Na^+ transporter. NHX/NHE transporters are from the large multi-gene CPA1 family and exist as multiple isoforms in most eukaryotes (Pardo et al. 2006), including six in *Arabidopsis* (Aharon et al. 2003). NHX proteins from class-I can catalyse Na^+/H^+ and K^+/H^+ exchange, where as class-II show a preference for K^+ over Na^+ (Pardo et al. 2006). Although membrane localisation has not been studied *in planta* here, *LtNHX1* is likely to be a vacuolar Na^+/H^+ exchanger, based on the following evidence: (1) Amino acid conservation of over 80% with other characterised *NHX1*-like plant genes; (2) Similar protein structure to other vacuolar NHX transporters, including 11 transmembrane spanning domains (Pardo et al. 2006); (3) A conserved amiloride-binding site also in the third transmembrane region (Putney et al. 2002); (4) A predicted cytosolic position of the C-terminus and vacuolar location of the N-terminus, as per *Arabidopsis* NHX1 and human isoform NHE1 (Sato and Sakaguchi 2005); and (5) *LtNHX1* functions in yeast similar to *Arabidopsis NHX1* (this study; Quintero et al. 2000). Although further experiments, such as measuring Na^+/H^+ exchange in isolated tonoplast vesicles (Apse and Blumwald 2007), would verify the identity of this gene; the evidence above suggests *LtNHX1* is a vacuolar Na^+/H^+ transporter, and therefore we propose is a candidate gene for maintaining Na^+ accumulation in root vacuoles during stagnant-plus-saline treatment.

The function of plant NHX transporters is often characterised using heterologous expression systems, which are particularly useful for non-model plants where the lack of available mutant lines can limit *in planta* assessment of gene function. For functional characterisation of *LtNHX1* we used the yeast strain AXT3, which is sensitive to salinity due to the removal of the endogenous plasma membrane Na^+ pump, ENA1-4, and antiporter, NHA1, as well as the endogenous vacuolar antiporter NHX1 (Gaxiola et al. 1999). Whereas wild-type yeast can tolerate NaCl concentrations above sea water (about 550 mM NaCl), growth of the AXT3 yeast mutant is completely inhibited at 100 mM NaCl (Fig. 7a). Heterologous expression of *LtNHX1* in AXT3 substantially improved its salt tolerance, with yeast growth now clearly visible at 100 mM (Fig. 7a). Cellular concentrations of K^+ did not differ between AXT3 and AXT3 transformed with *LtNHX1*, and Na^+ was only 10% lower with *LtNHX1*, which would not account for the significant improvement in salt tolerance (Fig. 7). Therefore, improved salt tolerance of AXT3 is likely due to LtNHX1 enabling accumulation of Na^+ in vacuoles under salt stress. Our results show *LtNHX1* has a similar function to *AtNHX1*, which also improved salt tolerance of AXT3 at 50 mM NaCl (Quintero et al. 2000). While we can still only speculate that a major role of *LtNHX1 in planta* is to accumulate Na^+ in

root vacuoles, the expression of *LtNHX1* in yeast has demonstrated that this gene can improve the salt tolerance of a unicellular system without a substantial decrease in cellular Na^+ (or increase in K^+). It would be interesting to determine if *LtNHX1* can also function as a K^+/H^+ exchanger and improve yeast growth at high external K^+.

It has been hypothesised that the expression of Na^+ and Cl^- transporters is impaired under combined saline and waterlogging stresses (e.g. Barrett-Lennard 2003); however, to our knowledge, this has never been experimentally tested. Using real-time qPCR, we studied gene expression of *LtNHX1* in roots of *L. tenuis* and *L. corniculatus* exposed to stagnant-plus-saline treatment for 48 h. Transcript levels of *LtNHX1* increased by 2 to 3-fold in response to aerated saline treatment for both species (Fig. 8). A similar induction of *NHX1* by salinity has been observed in several other species, including *Medicago* (Yang et al. 2005), soybean (Li et al. 2006), wheat (Brini et al. 2005), rice (Fukuda et al. 2004), *Suaeda salsa* (Ma et al. 2004, Zahran et al. 2007), *Thellungiella halophila* (Wu et al 2009) and *Populus euphratica* (Ye at al. 2009). For the stagnant-plus-saline treatment, transcript levels of *LtNHX1* in *L. tenuis* were similar to aerated saline treatment, demonstrating that the expression of this transporter was not impaired by combined salinity and waterlogging stresses. By contrast, *L. corniculatus* transcript levels for stagnant-plus-saline treatment were reduced by 3-fold relative to aerated saline treatment. We predict that the 4-fold higher expression of *LtNHX1* under stagnant-plus-saline treatment in roots of *L. tenuis*, compared with *L. corniculatus*, is due to enhanced root aeration, and may contribute to greater retention of Na^+ in roots. It is important to note that in addition to *NHX1*, root-zone O_2 deficiency could impair the function of the H^+ pumps V-ATPase and V-PPase, which would also affect Na^+ sequestration into root vacuoles under salt stress. However, transcripts of *HvHVA* and *HvHVP1* from barley (*Hordeum vulgare*) varieties that differed in salt tolerance showed no difference between the varieties after treatment with 100 mM NaCl, whereas *HvNHX1* transcript abundance was higher in the salt tolerant variety (Ligaba and Katsuhara 2010). It is not known if the combination of salinity and root-zone O_2 deficiency would affect the expression of vacuolar H^+ pumps, and future studies could determine what other transporters, in addition to *NHX1*, are impacted on by the adverse interaction between salinity and waterlogging.

In conclusion, the results of our study have demonstrated the importance of restricting root-to-shoot transport of Na^+ (and Cl^-) to improve tolerance to stagnant-plus-saline treatment. Tolerance of *Lotus tenuis* to combined salinity and waterlogging stresses is associated with a higher proportion of total plant Na^+ in the roots. Our data indicate that sustaining root aeration could enable *Lotus tenuis* to maintain expression of an *NHX1*-like gene, and thus contribute to accumulation of Na^+ into root vacuoles to reduce shoot Na^+ uptake under combined salinity and waterlogging.

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Tables

Table 1. Primer sequences used for cloning and quantitative real-time PCR of a *Lotus tenuis* *NHX1*-like gene.

Origin	Primer name	Sequence (5`→ 3`)	Amplicon (bp)	Anneal (°C)	Extension time (min)
Lotus japonicus	LjF LjR	TACTCATTGTGGAGGGTGGA TATGCGAGCTTGTACCACCA	250	52	0.5
Lotus tenuis	NQF NQR	TACTTCACTGCGGTCCAATG GATCTAGGGAAGCCATGCTG	150	52	0.5
Lotus tenuis	TQF TQR	AGGAAGGCTTTCTTGCATTG TCCTCCTGAACTTCATCCTCA	170	52	0.5

Table 2. Rates of net Na$^+$ transport and distribution of Na$^+$ between roots and shoots of saline and stagnant-plus-saline treated *Lotus tenuis* and *L. corniculatus*. Treatments were imposed on four-week-old plants for 14 d. Net transport rate is calculated between 7 and 14 d of treatment. Values are means (n=4) $\pm$ SE. Different letters within a column indicate significant differences (P<0.05) based on Tukey's test. N.B. There was no significant difference between the species in root:shoot mass ratio for any of the treatments.

Treatment	Species	Net rate of Na$^+$ transport to shoot (µmol g^{-1} root DW d^{-1})	Total plant Na$^+$ (µmol)	Root Na$^+$ content as % of total plant Na$^+$
Aerated, 0.1 mM NaCl	*Lotus tenuis*	9.4 $\pm$ 2.6 *a*	157 $\pm$ 12 *a*	92 $\pm$ 0.3 *a*
	Lotus corniculatus	13.2 $\pm$ 2.9 *a*	181 $\pm$ 39 *a*	91 $\pm$ 2.0 *a*
Stagnant, 0.1 mM NaCl	*Lotus tenuis*	7.4 $\pm$ 2.6 *a*	154 $\pm$ 8.4 *a*	94 $\pm$ 0.5 *a*
	Lotus corniculatus	7.9 $\pm$ 5.7 *a*	118 $\pm$ 16 *a*	94 $\pm$ 0.3 *a*
Aerated, 200 mM NaCl	*Lotus tenuis*	345 $\pm$ 42 *b*	755 $\pm$ 48 *b*	59 $\pm$ 3.4 *b*
	Lotus corniculatus	483 $\pm$ 25 *bc*	1230 $\pm$ 280 *b*	57 $\pm$ 2.0 *b*
Stagnant, 200 mM NaCl	*Lotus tenuis*	607 $\pm$ 17 *c*	832 $\pm$ 72 *b*	55 $\pm$ 2.3 *b*
	Lotus corniculatus	786 $\pm$ 40 *d*	903 $\pm$ 153 *b*	39 $\pm$ 1.7 *c*

Figure legends

Figure 1. Rates of radial O_2 loss (ROL) along primary lateral roots of *Lotus tenuis* and *L. corniculatus* when in an O_2-free solution with shoots in air. Four-week-old plants were exposed to stagnant root-zone treatments. Measurements were taken after 8 d of stagnant treatment. Average root lengths were 59 ± 3.9 for *L. corniculatus* and 82 ± 5.3 for *L. tenuis*. Values are means ($n=4$) $\pm$ SE. Significant differences ($P \leq 0.05$, based on Tukey's HSD test) between *L. tenuis* and *L. corniculatus* (stagnant treatment only) are indicated by *.

Figure 2. O_2 consumption rates of excised 2 cm tips of lateral roots from *Lotus tenuis* and *L. corniculatus*. Four-week-old plants were exposed to 0.1 mM NaCl or 200 mM NaCl aerated root-zone treatments for 14 d. O_2 consumption was measured at 20°C in nutrient solution containing either 0 or 200 mM NaCl. Values are means ($n=4$) $\pm$ SE. There was no significant differences between species or treatments ($P \geq 0.05$).

Figure 3. Concentrations of Cl^-, Na^+ and K^+ in roots and shoots of saline and stagnant-plus-saline treated *Lotus tenuis* and *L. corniculatus*. Four-week-old plants were exposed to either aerated 200 mM NaCl (A, C, E) or stagnant 200 mM NaCl (B, D, F) root-zone treatments, imposed as indicated in the graph. NaCl was added in daily 50 mM increments. Measurements were taken on days 0, 7 and 14 during the treatment period. Values are means ($n=4$) $\pm$ SE. Significant differences ($P \leq 0.05$, based on Tukey's HSD test) between *L. tenuis* and *L. corniculatus* are indicated by * for shoots and # for roots. Note all axes have the same scale. Values in non-saline treatments (data not shown) did not differ significantly from concentrations at day 0 in the graph.

Figure 4. Relative growth rate (RGR) of whole plants for *Lotus tenuis* and *L. corniculatus* in saline and stagnant-plus-saline root-zone treatments. Four-week-old plants were exposed to control (aerated, 0.1 mM NaCl), stagnant (0.1 mM NaCl), saline (aerated, 200 mM NaCl) or stagnant-plus-saline (200 mM NaCl) root-zone treatments. RGR was calculated from whole plant dry weights measured after 7 and 14 d NaCl treatments (1 and 8 d stagnant treatment,

respectively). Values are means (n=4) $\pm$ SE. Different letters indicate significant differences (P<0.05) based on Tukey's test.

Figure 5. Sequence analysis of a *Lotus tenuis NHX1*-like gene. **A:** Putative membrane topology of LtNHX1 protein (predicted by hydropathy profiles using ConPredII) showing the 11 transmembrane domains at the respective amino acid residues. **B:** Phenogram (CLUSTALW) of LtNHX1 compared to AtNHX1-6 proteins showing LtNHX1 is most similar to AtNHX1. Values in parentheses indicate similarity to LtNHX1 at the nucleotide level. **C:** Phenogram (CLUSTALW) showing similarity at the nucleotide level of *LtNHX1* to a selection of other *NHX1*- like genes from dicotyledonous species.

Figure 6. Alignment of a *Lotus tenuis NHX1*-like gene to other *NHX1*-like genes using CLUSTALW. A predicted amiloride-binding site is underlined in *L. tenuis.*

Figure 7. Functional complementation of *LtNHX1* in yeast. The yeast strain AXT3 (lacking endogenous Na$^+$ transporters ENA1-4, NHX1, NHA1) was transformed with *LtNHX1* and compared with wild type yeast (WT). Overnight cultures in minimal media were incubated in YPGAL until OD$_{600}$ of 0.6. **A:** Cultures were serially diluted from 10^{-1} to 10^{-3} and 5 µl of each dilution was pipetted onto YPGAL plates with 0, 75 and 100 mM NaCl. Plates were incubated at 30°C for 4 d. Drop tests were repeated for three individual cultures of each strain from two independent transformations and the same results obtained. **B:** Cultures were re-suspended in YPGAL plus 100 mM NaCl for 8 h and concentrations of Na$^+$ and K$^+$ in the cells measured. Values are means ($\pm$SE) of four individual cultures. Different letters (P<0.05) indicate significant differences between strains for each ion based on Tukey's test.

Figure 8. Expression of *LtNHX1*–like gene in roots of saline and saline-plus-stagnant treated *Lotus tenuis* and *L. corniculatus*. Four-week-old plants were exposed to control (aerated, 0.1 mM NaCl), stagnant (0.1 mM NaCl), saline (aerated, 200 mM NaCl) or stagnant-plus-saline (200 mM NaCl) root-zone treatments. RNA was extracted from root tissue harvested after 7 d NaCl treatments (1 d stagnant treatment). Stagnant treatment was not significantly different from control for both species (data not shown). **A:** Real-time qPCR results. Values are means (n=3) $\pm$

SE. Samples were run in duplicate and each run repeated. Data were normalised using qBase software and *LtTUB1* as a reference gene. * indicates a significant difference (P<0.05) between *L. tenuis* and *L. corniculatus* for that treatment (Tukey's test). **B:** qPCR samples of *L. tenuis* (*Lt*) and *L. corniculatus* (*Lc*) were run on 2.5% agarose gel at 80 V for 30 min. A representative gel is shown and similar results were obtained for all replicates.

Figures

Fig. 1

Fig. 2

Fig. 3

Fig. 4

A.

B.

AtNHX2   (71%)

AtNHX1   (83%)

LtNHX1

AtNHX3

AtNHX4

AtNHX6

AtNHX5

C.

Thellungiella halophilia

Arabidopsis thaliana

Atriplex dimorphostegia

Suaeda maritima

Trifolium repens

Lotus tenuis

Glycine max

Fig. 5

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              Lotus tenuis  ---MGLGLISAVS-KLQMVSTSDHASIVSMNLFVALLCACIVLGHLLEENRWMNESITALVIGVCVGVVILLFSG
           Trifolium repens  ---MAIEIASVVS-KLQMLSTSDHSSVVSMNLFVALLCACIVLGHLLEENRWMNESITALLIGIATGVVILLLSG
     Thellungiella halophila  MAMLASYFDSFIS-KMPSLSTSDHASVVSLNLFVALLCACIVLGHLLEENRWMNESITALLIGLATGVVILLISK
     Atriplex dimorphostegia  ---MWSQLSSLLSEKMDALATSDHASVVSMNLFVALLCGCIVIGHLLEENRWMNESITALLIGLSTGVVILLISG
             Suaeda maritima  ---MWSQLSSFFASKMDMVSTSDHASVVSMNLFVALLCGCIVIGHLLEENRWMNESITALLIGLSTGIIILLISG
         Arabidopsis thaliana  ------MLDSLVS-KLPSLSTSDHASVVALNLFVALLCACIVLGHLLEENRWMNESITALLIGLGTGVTILLISK
                 Glycine max  ---MGFEISTVVS-KLQTLSTSDHASVVSMNLFVALLCGCIVLGHLLEENRWMNESITALLIGVCTGIVILLFSG
                              :  :  .:  *:    ::****:*:*::*********.***:*****************:**: .*: ***:*-

              Lotus tenuis  GTSSHILVFSEDLFFIYLLPPIIFNAGFQVKKKQFFVNFMTITMFGAIGTLISCTIITLGATQAFKWMDVGPLEV
           Trifolium repens  GKSSHILVFSEDLFFIYLLPPIIFNAGFQVKKKQFFVNFMIIASFGAIGTLISCVVITFGATQAFKMMDIGPLDI
     Thellungiella halophila  GKSSHLLVFSEDLFFIYLLPPIIFNAGFQVKKKQFFRNFVTIMLFGAIGTVISCTVITLGVTQFFKKLDIGTFDL
     Atriplex dimorphostegia  GKSSHLLVFSEDLFFIYLLPPIIFNAGFQVKKKQFFRNFITIVLFGAVGTLVSFTIISLGALSIFKKLDIGTLEL
             Suaeda maritima  GKSSHLLVFSEDLFFIYLLPPIIFNAGFQVKKKQFFRNFITIILFGAVGTLVSFIIISLGSIAIFQKMDIGSLEL
         Arabidopsis thaliana  GKSSHLLVFSEDLFFIYLLPPIIFNAGFQVKKKQFFRNFVTIMLFGAVGTIISCTIISLGVTQFFKKLDIGTFDL
                 Glycine max  GKSSHILVFSEDLFFIYLLPPIIFNAGFQVKKKQFFVNFMTIMLFGAIGTLISCTIITLGATQIFKRLDVGPLEL
                              *.***:**************************** **: *   ***:**::*  :*::*     *: :*:*.::: 

              Lotus tenuis  GDLLAIGAIFAATDSVCTLQVLNQDETPLLYSLVFGEGVVNDATSVVLFNAIQSFDLNQINS----SIALHFLGN
           Trifolium repens  GDYLAIGAIFAATDSVCTLQVLNQDETPLLYSLVFGEGVVNDATSVVLFNAIQSFDLDRLNP----SIAVHLLGN
     Thellungiella halophila  GDYLAIGAIFAATDSVCTLQVLNQDETPLLYSLVFGEGVVNDATSVVVFNAIQSFDLTHLNH----EAAFHLLGN
     Atriplex dimorphostegia  ADYLAIGAIFAATDSVCTLQVLNQDETPLLYSLVFGEGVANDATSVVLFNAIQSFDLTRIDH----RIALQFMGN
             Suaeda maritima  GDLLAIGAIFAATDSVCTLQVLNQDETPLLYSLVFGEGVVNDATSVVLFNAIQNFDLTHIDHRIAYRIAFQFGGN
         Arabidopsis thaliana  GDYLAIGAIFAATDSVCTLQVLNQDETPLLYSLVFGEGVVNDATSVVVFNAIQSFDLTHLNH----EAAFHLLGN
                 Glycine max  GDFLAIGAIFAATDSVCTLQVLNQDETPLLYSLVFGEGVVNDATSVVLFNAIQSFDLNQIDP----SIAGHFLGN
                              .*  *******************************.*******:*****.*** :::        *  :: **

              Lotus tenuis  FLYLFIASTLLGVLTGLLSAYIIKKLYIGRHSTDREVALMMLMAYLSYMLAELAYLSGILTVFFCGIVMSHYTWH
           Trifolium repens  FLYLFIASTLLGVLTGLLSAYVIKKLYIGRHSTDREVALMMLMAYLSYMLAELFYLSGILTVFFCGIVMSHYTWH
     Thellungiella halophila  FLYLFLLSTLLGVATGLISAYVIKKLYFGRHSTDREVALMMLMAYLSYMLAELFDLSGILTVFFCGIVMSHYTWH
     Atriplex dimorphostegia  FSYLFIASTILGAFTGLLSAYVIKKLYFGRHSTDREVALMMLMAYLSYMLAELFYLSGILTVFFCGIVMSHYTWH
             Suaeda maritima  FLYLFFASTLLGAVTGLLSAYVIKKLYFGRHSTDREVALMMLMAYLSYMLAELFYLSGILTVFFCGIVMSHYTWH
         Arabidopsis thaliana  FLYLFLLSTLLGAATGLISAYVIKKLYFGRHSTDREVALMMLMAYLSYMLAELFDLSGILTVFFCGIVMSHYTWH
                 Glycine max  FLYLFIASTMLGVLTGLLSAYIIKKLYIGRHSTDREVALMMLMAYLSYMLAELSYLSGILTVFFCGIVMSHYTWH
                              *.*** :**:**: ***.***:****:*****************************   *******************
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              Lotus tenuis  NVTESSRITTKHAFATLSFVAETFIFLYVGMDALDIEKWKFVSDSPGTSVAVSSVLLGLILLGRAAFVFPLSFLS
           Trifolium repens  NVTESSRVTTKHSFATLSFVAEIFIFLYVGMDALDIEKWKFVSDSPGTSIATSAVLLGLILLGRAAFVFPLSFLS
      Thellungiella halophila  NVTESSRITTKHTFATLSFLAETFIFLYVGMDALDIDKWRSVSDSPGTSVAVSSILIGLLMLGRAAFVFPLSFLS
      Atriplex dimorphostegia  NVTESSRVTTKHAFATLSFVAEVFLFLYVGMDALDIEKWRFVSDSPGISVAVSSILLGLVMVGRAAFVFPLSWLM
             Suaeda maritima  NVTESSRVTTKHAFATLSFVAEIFIFLYVGMDALDIEKWRFVSDSPGTSVAVSSILLGLLMVGR-ALLFSLVFLM
          Arabidopsis thaliana  NVTESSRITTKHTFATLSFLAETFIFLYVGMDALDIDKWRSVSDTPGTSIAVSSILMGLVMVGRAAFVFPLSFLS
                  Glycine max  NVTESSRITTKHSFATLSFVAEIFIFLYVGMDALDIEKWKFVSDSPGTSVATSSVLLGLILLGRAAFVFPLSFLS
                               *******:****:*******:** *:************:**: ***:** *:*.*::*:**:::** *::*.* :*.

              Lotus tenuis  NLAKKSQNDKISFRQQVIIWWAGLMRGAVSMALAYNQFTMSGHTSLRSNAIMITSTITVVLVSTVVFGMLTKPLI
           Trifolium repens  NLTKKSQHQKISFRQQVIIWWAGLMRGAVSMALAYNQFTRSGHTQLRSNAIMITSTITVVLFSTVVFGLLAKPLI
      Thellungiella halophila  NLAKKNESEKINFKMQVVIWWAGLMRGAVSMALAYNKFTRAGHTDLRGNAIMITSTITVCPFSTVVFGMLTKPLI
      Atriplex dimorphostegia  NLAKKSHGEKVTFNQQIVIWWAGLMRGAVSMALAYNQFTRSGHTQLRGNAIMITSTISVVLFSTMVFGLLTKPLI
             Suaeda maritima  NLSKKSNSEKVTFNQQIVIWWAGLMRGAVSVALAYNQFSRSGHTQLRGNAIMITSTITVVLFSTMVFGLLTKPLI
          Arabidopsis thaliana  NLAKKNQSEKINFNMQVVIWWSGLMRGAVSMALAYNKFTRAGHTDVRGNAIMITSTITVCLFSTVVFGMLTKPLI
                  Glycine max  NLAKKSPNEKISFRQQVIIWWAGLMRGAVSIALAYNQFTMSGHTSLRSNAIMITSTITVVLFSTVVFGLLTKPLI
                               **:**.  :*:.*. *::***:*******:*****:*: :***.:*.*********:*  .**:***:*:****

              Lotus tenuis  RLLLPHTDTKTDS--MASLDPSTP--KSFTVPLLGSAQDSVGDFDA---------HEIRRPSSIRALLSTPTHTV
           Trifolium repens  RLLLPHQKATNS---MTTTESSTP--KSVIVPLLGEPRDSEADLEG---------HEIHRPNSIRALLSTPTHTV
      Thellungiella halophila  RHLLPHQKATTS---MLSGDNNTP--KSIQIPLLD--QDSFIEFAGN--------HNVPRPDSIRGFLTRPTRTV
      Atriplex dimorphostegia  MFLLPHPKHFTS--CSTVSDVGSP--KSYSLPLLEGNQDYEVDVGNGNHEETTEQRTIVRPSSLRMLLNAPTHTV
             Suaeda maritima  LFMLPQPKHFTS--ASTVSDLGSP--KSFSLPLLEDRQDSEADLGN-DDEEAYPRGTIARPTSLRMLLNAPTHTV
          Arabidopsis thaliana  SYLLPHQNATTS---MLS-DDNTP--KSIHIPLLD--QDSFIEPSGN--------HNVPRPDSIRGFLTRPTRTV
                  Glycine max  RLLLPHTPHHKESSITIITDPSTPSLKSVTIPLLGSAQESEVDIDG---------HDIHRPSSIRALLTTPTHTV
                               :**:    ..       : .:*  **  :***  ::   :            : ** *:* :*. **:**

              Lotus tenuis  HRLWRKFDNSFMRPVFGGRGFVPVEPGSPSDRSNGHQWH--
           Trifolium repens  HRLWRKFDDSFMRPVFGGRGFVPVEPGSPTER-NGNLWR--
      Thellungiella halophila  HYYWRQFDDSFMRPVFGGRGFVPFVPGSPIERDPPDLSKA-
      Atriplex dimorphostegia  HHYWRKFDDSFMRPVFGGRGFVPFVPGSPTEQSTNNLVDGT
             Suaeda maritima  HHYWRRFDDYFMRPVFGGRGFVPFVPGSPTEQSTTNLSQRT
          Arabidopsis thaliana  HYYWRQFDDSFMRPVFGGRGFVPFVPGSPTERNPPDLSKA-
                  Glycine max  HRLWRKFDDAFMRPVFGGRGFVPVEPGSPTER-NGHQWR--
                               *  **:**: *************.**** ::         .
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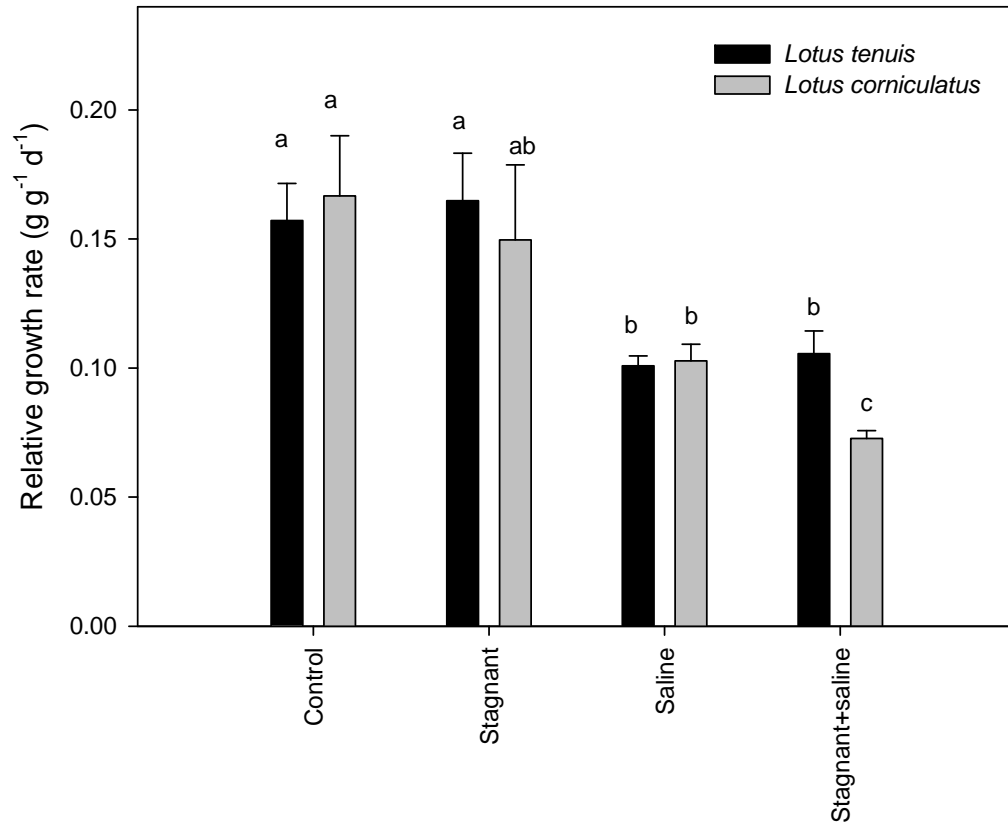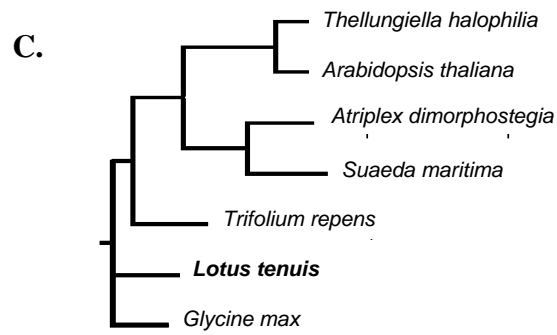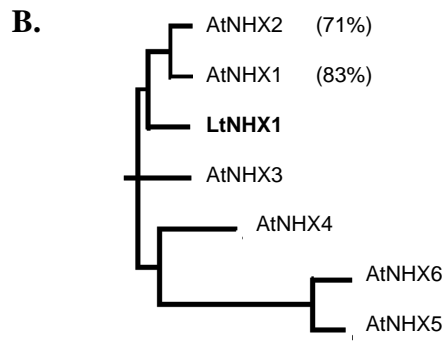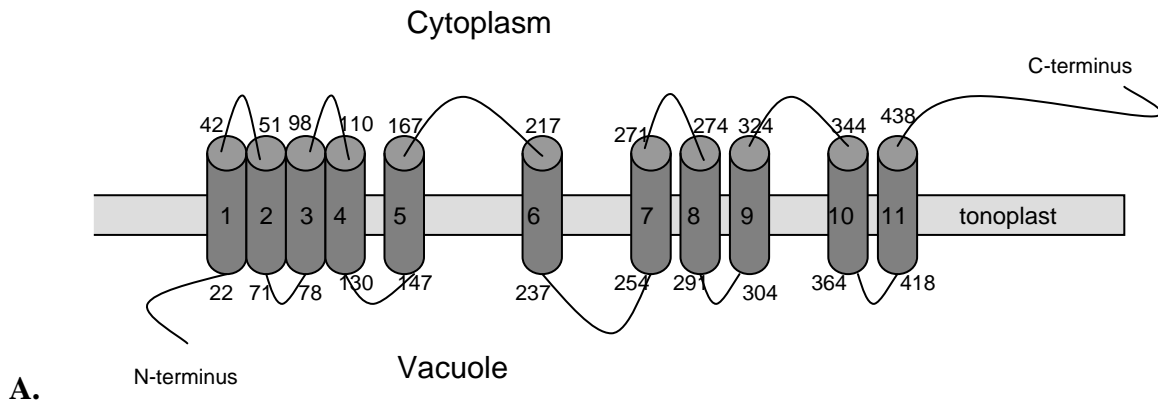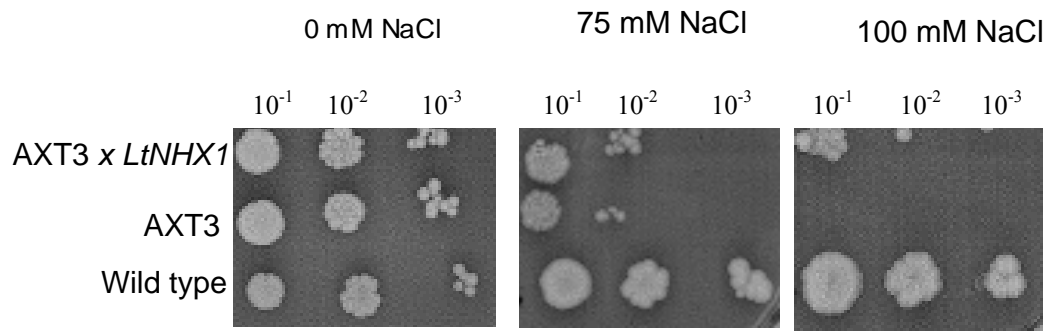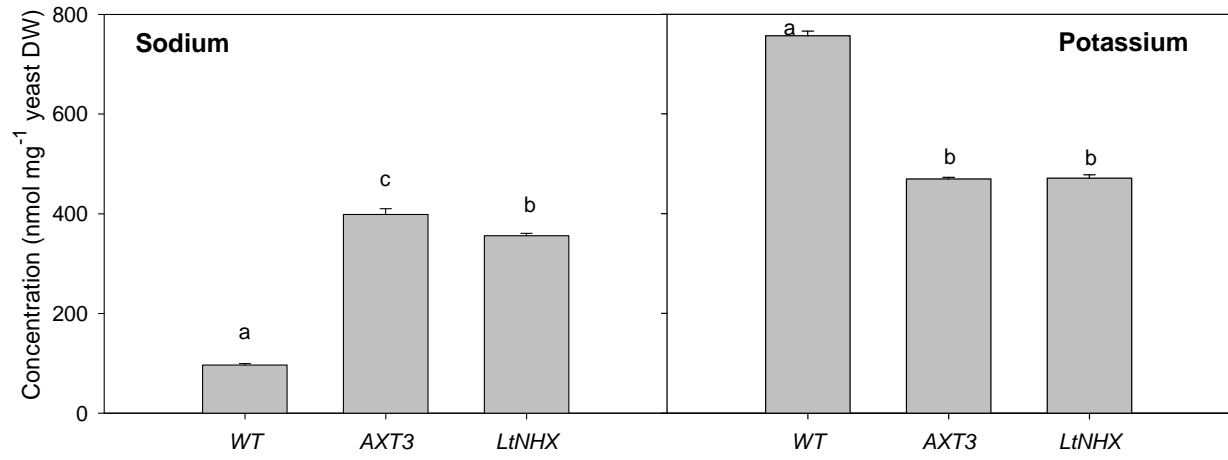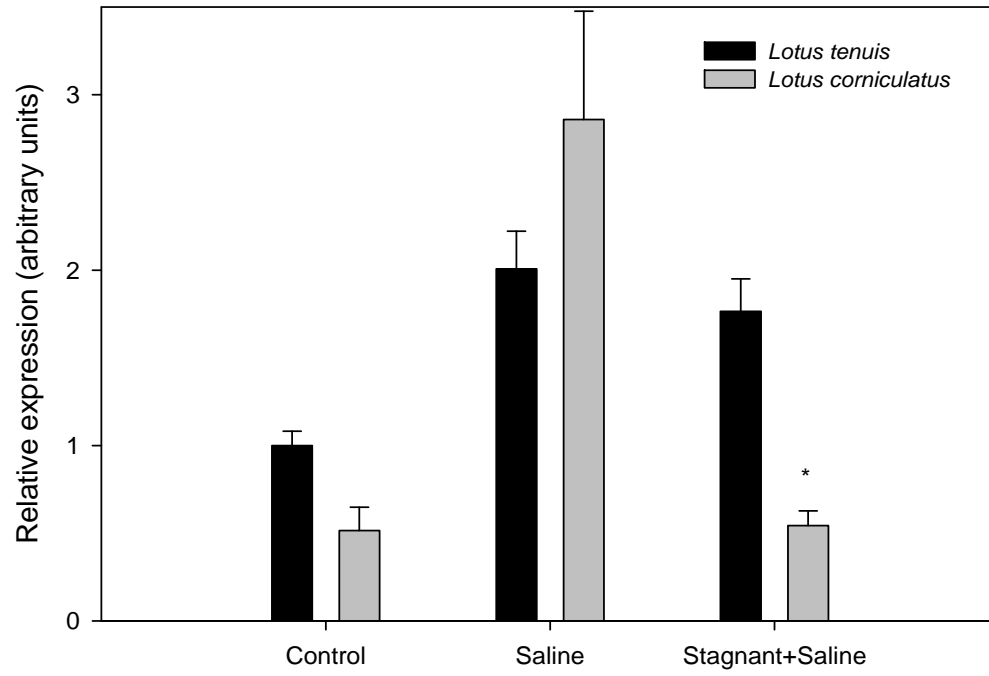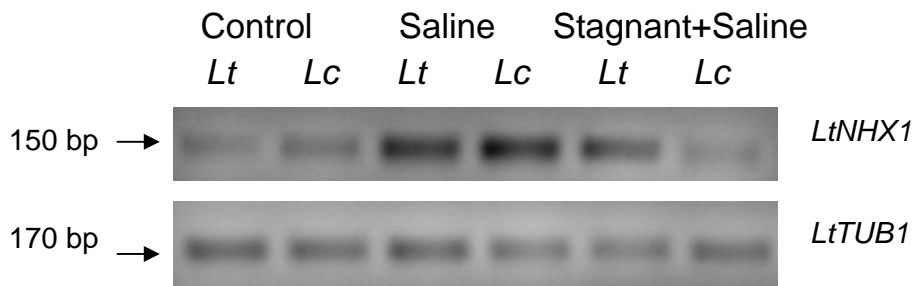
┌───┐
│ ***** - single, fully conserved residue │
│ **:** - conservation of strong groups   │
│ **.** - conservation of weak groups   │
│ - no consensus   │
└───┘

Fig. 6

A

B

Fig. 7

A

B

Fig. 8