

Evidence that differential gene expression between the halophyte, *Thellungiella halophila*, and *Arabidopsis thaliana* is responsible for higher levels of the compatible osmolyte proline and tight control of Na⁺ uptake in *T. halophila*

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ABSTRACT

Salt-sensitive glycophytes and salt-tolerant halophytes employ common mechanisms to cope with salinity, and it is hypothesized that differences in salt tolerance arise because of changes in the regulation of a basic set of salt tolerance genes. We explored the expression of genes involved in two key salt tolerance mechanisms in *Arabidopsis thaliana* and the halophytic *A. thaliana* relative model system (ARMS), *Thellungiella halophila*. Salt overly sensitive 1 (SOS1) is a plasma membrane Na⁺/H⁺ antiporter that retrieves and loads Na⁺ ions from and into the xylem. Shoot *SOS1* transcript was more strongly induced by salt in *T. halophila* while root *SOS1* was constitutively higher in unstressed *T. halophila*. This is consistent with a lower salt-induced rise in *T. halophila* xylem sap Na⁺ concentration than in *A. thaliana*. *Thellungiella halophila* contained higher unstressed levels of the compatible osmolyte proline than *A. thaliana*, while under salt stress, *T. halophila* accumulated more proline mainly in shoots. Expression of the *A. thaliana* ortholog of proline dehydrogenase (PDH), involved in proline catabolism, was undetectable in *T. halophila* shoots. The PDH enzyme activity was lower and *T. halophila* seedlings were hypersensitive to exogenous proline, indicating repression of proline catabolism in *T. halophila*. Our results suggest that differential gene expression between glycophytes and halophytes contributes to the salt tolerance of halophytes.

Key-words: real-time PCR; salt stress; salt overly sensitive 1; proline dehydrogenase; Δ^1 -pyrroline-5-carboxylate synthetase.

INTRODUCTION

Salt stress, caused by high concentrations of salts in the soil, is a prime factor limiting crop productivity worldwide, and

along with other abiotic stresses, is estimated to decrease the average yields of the major crop plants by over 50% (Bray, Bailey-Serres & Weretilnyk 2000). The severity of the problem is illustrated by the fact that one-third of global arable land is significantly affected by both man-made and naturally occurring salinization (Munns 2002; Eimer 2004).

One of the earliest effects on plants exposed to salinity is on plant water status (Bray *et al.* 2000; Munns 2002). The reduction in soil water potential caused by increased soil solutes makes it increasingly difficult to maintain a gradient of water flow into the plant. The resulting osmotic stress leads to stomatal closure in some plant species (Lawlor 1995), a reduced rate of photosynthesis (Ort, Oxborough & Wise 1994; Chaves *et al.* 2002) and a reduction in plant growth partly caused by the decrease in carbon assimilation but also as a result of direct inhibition of cell division and expansion (Zhu 2001; Munns 2002). Salinity also causes disruption of ionic homeostasis which can lead to the accumulation of toxic ions such as Na⁺ and Cl⁻ in the cells, thereby adversely affecting cell membrane integrity, enzyme activities, nutrient acquisition and the function of the photosynthetic apparatus (Zhu 2001; Tester & Davenport 2003). A secondary effect of high salinity is the production of reactive oxygen species (ROS) that are highly destructive to lipids, nucleic acids and proteins (Burdon *et al.* 1996; Asada 1997; Tsugane *et al.* 1999; Xiong & Zhu 2002a).

Plants possess a number of mechanisms to prevent accumulation of Na⁺ in the cytoplasm that include minimizing Na⁺ influx, intracellular compartmentation of Na⁺ and maximizing Na⁺ efflux via Na⁺/H⁺ antiporters in the plasma membrane as well as recirculation of Na⁺ out of the shoot via the phloem (Apse *et al.* 1999; Perez-Alfocea *et al.* 2000; Shi *et al.* 2002; Ward, Hirschi & Sze 2003). Vacuolar compartmentation of Na⁺ also has the effect of lowering cell water potential, thereby sustaining water absorption from the soil (Apse *et al.* 1999; Hasegawa *et al.* 2000; Gaxiola *et al.* 2001). However, the lower osmotic potential in the vacuole needs to be balanced with that of the cytoplasm, and this is achieved by the accumulation of non-toxic (compatible) osmolytes in the cytosol including sugars, polyols

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and amino acids (Xiong & Zhu 2002b). Among compatible osmolytes, proline accumulates in many plant species in response to abiotic stress (Delauney & Verma 1993). In addition to reducing cytosolic osmotic potential, proline has been reported as having a role in protecting proteins against denaturation (Rajendrakumar, Reddy & Reddy 1994) and scavenging ROS (Smirnov & Cumbes 1989; Hong *et al.* 2000). Indeed, prevention of cellular damage caused by ROS is an important stress tolerance trait. Elimination of ROS is mainly undertaken by antioxidant compounds and by ROS-scavenging enzymes (Bartley & Scolnik 1995; Dixon & Paiva 1995; Asada 1997; Chalker-Scott 1999; Xiong & Zhu 2002a; Apel & Hirt 2004).

Underlying the physiological responses to salt stress are changes in gene expression. Thus, salinity affects the transcriptional level of approximately 13% of the *Arabidopsis thaliana* genome (Kreps *et al.* 2002). These genes encode proteins involved in numerous biological processes and stress responses as well as a large number of proteins of unknown function. Importantly, many of the affected genes such as transcription factors and kinases may function in salt stress-responsive regulatory circuits (Chen *et al.* 2002; Kreps *et al.* 2002; Seki *et al.* 2002). Indeed, it has been hypothesized that differences in salt tolerance mechanisms between salt-sensitive glycophytes, such as *A. thaliana*, and salt-tolerant halophytes, result from changes in the regulation of the same basic set of genes involved in salt tolerance (Zhu 2000; Zhu 2001; Xiong & Zhu 2002a).

The recent characterization of the halophyte *Thellungiella halophila* (salt cress) as an *A. thaliana* relative model system (ARMS) (Bressan *et al.* 2001; Inan *et al.* 2004; Volkov *et al.* 2004) is allowing the question of differential expression of salt tolerance genes between glycophytes and halophytes to be addressed using the molecular and genetic tools available for *A. thaliana*. Thus, Taji *et al.* (2004) employed an *A. thaliana* full-length cDNA microarray comprising approximately 7000 genes to compare the expression profiles of *A. thaliana* and *T. halophila* under unstressed and salt-stress conditions. They reported that many genes known to be induced by abiotic stress in *A. thaliana* exhibit higher levels of expression in *T. halophila* under unstressed conditions, suggesting that constitutive expression of a conserved stress machinery forms a basis for the salt tolerance of *T. halophila*. Gong *et al.* (2005) used a 25 000-element microarray to compare *A. thaliana* and *T. halophila* transcript profiles. They observed that the two species exhibited both shared and divergent responses to salt stress with many of the *A. thaliana*-specific responses representing injury-based defense reactions. A comparison of *A. thaliana* and *T. halophila* transcript intensities suggested stress-anticipatory preparedness in *T. halophila*.

One problem in directly comparing inter-species mRNA levels is that a lower hybridization signal in *T. halophila* compared to *A. thaliana* could either be caused by a lower expression level or to lower hybridization efficiency because of probe mismatches. A similar problem can exist

when using real-time PCR with *A. thaliana* gene-specific primers.

In the present study, we used *A. thaliana* gene-specific primers that showed similar real-time PCR amplification efficiencies with both *A. thaliana* and *T. halophila* cDNA to further explore how the expression of specific salt tolerance orthologues differs between unstressed and stressed plants of both species.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana (Columbia ecotype) and *T. halophila* (Shandong ecotype) seeds were surface-sterilized with 50% commercial bleach, washed three times in sterile water and sown on Murashige and Skoog (MS) agar plates (Murashige & Skoog 1962). The plates were placed at 4 °C for 4 d to synchronize germination, and then moved to a controlled growth room at 22 °C with a 16/8 h light/dark photoperiod and light intensity of 150 $\mu\text{E m}^{-2} \text{s}^{-1}$. Five-day-old seedlings were transferred to a 1:1 mixture of perlite (three mesh) and soil (*A. thaliana* growing medium PM-05; Lehle Seeds Co., Round Rock, TX, USA). This soil mixture allows leaching of nutrient solution to prevent build-up of salts and also permits easy harvesting of roots. The plants were irrigated with one-third Hoagland's nutrient solution (Hoagland & Arnon 1950). Because the initial growth of *T. halophila* is slightly slower than *A. thaliana*, the salt treatments were applied 5 d after the transplanting of *A. thaliana* seedlings and 2 d later for *T. halophila* seedlings. This ensured that salinity was applied at a similar physiological stage. Salt treatments commenced with 15% of the respective final NaCl concentration to avoid salt/osmotic shock in treatments below 500 mM NaCl. Salt concentrations were increased incrementally by 15% each day to reach final NaCl concentrations of 0, 100, 250, 500 and 750 mM NaCl at the same plant developmental stage for each treatment. One week after the final NaCl concentration was reached, the rosette leaf number was recorded and tissue was harvested. Roots and shoots were separated, fresh weight (FW) calculated and subsamples dried for 72 h at 65 °C for dry weight (DW) and ion analysis. The remaining tissue was snap frozen in liquid nitrogen and stored at -80 °C until use.

Anthocyanin content

The relative anthocyanin content was analysed based on a modified protocol according to Neff & Chory (1998). Approximately 50 mg of fresh shoot tissue was incubated overnight in 1 mL methanol (acidified with 1% HCl). Anthocyanins were separated from chlorophylls by adding 700 μL distilled water and extracting with 1.75 mL chloroform followed by centrifugation at 4000 g for 2 min. The total anthocyanins in the aqueous phase was determined by measuring the A_{530} and A_{657} . The relative amount of antho-

cyanin was calculated by subtracting the A_{657} from the A_{530} , and was expressed on a per gram FW basis.

Tissue Na⁺ and K⁺ analysis

Dry tissue (100 mg) was ground to a fine powder and digested overnight with 2 mL H₂SO₄, followed by heating at 150 °C for 1 h. H₂O₂ (0.5 mL) was added to the extract, which was reheated to 240 °C for 1 h. A further 0.5 mL of H₂O₂ was added to the extract, and the solution was reheated to 240 °C for 20 min; this step was repeated until clarification of the solution. The solution was diluted 50 times with distilled water, and Na⁺ and K⁺ contents were determined by atomic absorption spectrophotometry (Perkin-Elmer 1100B spectrophotometer, Norwalk, CT, USA).

Xylem sap Na⁺ analysis

Twenty-five-day-old *A. thaliana* and 28-day-old *T. halophila* plants were irrigated with one-third Hoagland's nutrient solution with or without 100 mM NaCl for 24 h. Xylem sap collection was performed according to Shi *et al.* (2002). The sap collected from three to six plants was pooled and diluted directly in distilled water, and Na⁺ concentration was measured using a Corning 410 flame photometer (Corning, Acton, MA, USA).

Proline content

The proline content was measured according to Bates, Waldren & Teare (1973). Tissue (250 mg) was homogenized with 10 mL of 3% sulphosalicylic acid and centrifuged at 2000 g for 5 min. Two millilitres of acid-ninhydrin and 2 mL of glacial acetic acid were added to 2 mL of supernatant, and the reaction was incubated for 1 h at 100 °C. Proline was extracted with 4 mL of toluene and proline concentration was assessed by measuring A_{520} of the toluene phase. The proline content was calculated using standard dilutions of L-proline and expressed on an FW basis.

Proline dehydrogenase (PDH) enzyme activity

The PDH activity was assayed according to Lutts, Majerus & Kinet (1999). The frozen plant tissue was homogenized in a pre-chilled mortar and pestle with acid-washed sand and enzyme extraction buffer [50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.4), 7 mM MgCl₂, 0.6 M KCl, 3 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF), 5% (w/v) polyvinylpyrrolidone]. The extracts were centrifuged at 16 000 g for 10 min and the supernatant was used for enzyme assays. The activity was assayed in 0.15 M Na₂CO₃-HCl buffer (pH 10.3) containing 1.5 mM NAD⁺ and 15 mM L-proline. The reduction of NAD⁺ was monitored at 340 nm. The PDH activity is expressed as nanomoles of NAD per minute per milligram of protein. The protein content was measured according to Bradford (1976).

Quantitative real-time PCR

RNA isolation and cDNA preparation

Total RNA was isolated from the shoot and root tissue using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) (http://www.arabidopsis.org/info/2010_projects/comp_proj/AFGC/RevisedAFGC/site2RnaL.htm#isolation) according to the manufacturer's protocol (<http://www.invitrogen.com/content/sfs/manuals/15596026.pdf>). To eliminate residual genomic DNA, 5 µg of total RNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA) according to the manufacturer's instructions. The total RNA was quantified with a NanoDrop spectrophotometer (ND-1000; NanoDrop Technologies, Wilmington, DE, USA) and its integrity was checked by loading on a denaturing 1% (w/v) agarose gel. cDNA was synthesized from 1 µg of total RNA by using the GeneAmp RNA PCR Kit (Applied Biosystems, Foster City, CA, USA) according to the supplier's instructions.

Primer design

Primers for amplification of PCR products of between 50 and 120 bp from *A. thaliana* and *T. halophila* cDNA were designed using *A. thaliana* sequences from Genbank and the Primer Express 2.0 software (Applied Biosystems). *Thellungiella halophila* PCR products for each ortholog were examined for a similar-sized PCR product as amplified from the respective *A. thaliana* gene and sequenced to ensure they encoded the expected gene product. The sequences of each primer pair are as follows:

Salt overly sensitive 1 (SOS1) F, 5'-CCTTACACTGTCGCTCTTCTCGTTA-3'
SOS1 R, 5'-TTAGCTCCATATTCGAGAGATCCA-3'
 Δ^1 -Pyrroline-5-carboxylate synthetase 1 (*P5CS1*) F, 5'-GAGCTAGATCGTTCACGTGCTTT-3'
P5CS1 R, 5'-ACAACACTGCTGTCCCAACCTTAAC-3'
 Δ^1 -Pyrroline-5-carboxylate synthetase 2 (*P5CS2*) F, 5'-GTTAAGCGTATCGTCGTCAAGTT-3'
P5CS2 R, 5'-CCTAAACGTCCAAGAGCCAATCT-3'
Ornithine- δ -aminotransferase (OAT) F, 5'-CTCAACGATTGATGGAATTGGA-3'
OAT R, 5'-ACCGGAACAGGATGGTAATTGT-3'
proline dehydrogenase (PDH) F, 5'-TCACAACCACTGAGCTAAAGTGAGA-3'
PDH R, 5'-CGATGACGCTGTATCTTGTGATG-3'
Ubiquitin 10 (UBQ10) F, 5'-CTCTCTACCGTGATCAAGATGCA-3'
UBQ10 R, 5'-TGATTGTCTTCCGGTGAGAGTC-3'

Real-time PCR conditions and analysis

Real-time PCR was performed with an ABI PRISM 7500 Sequence Detection System (SDS) (Applied Biosystems) using SYBR Green (Applied Biosystems) to monitor double-stranded DNA (dsDNA) synthesis. Each reaction contained 10 µL SYBR Green master mix reagent (Applied Biosystems), 40 ng cDNA and 500–700 nm of gene-specific

primer in a final volume of 20 μ L. The optimum primer concentration was checked by template amplifications in the range 300–700 nM for each primer. PCR amplifications were performed using the following thermal profile: 95 °C for 10 min (hot start), 40 cycles of 95 °C for 15 s (denaturation) and 60 °C for 1 min (annealing/extension). Data were analysed using the SDS 1.2 software (Applied Biosystems). To check the specificity of annealing of the primers, dissociation kinetics was performed at the end of each PCR run. All reactions were performed in triplicates from three independent experiments. All amplification plots were analysed with a normalized reporter (Rn) threshold of 0.2 to obtain threshold cycle (C_T) values. The relative quantification (RQ) values for each target gene were calculated by the $2^{-\Delta\Delta C_T}$ method using *UBQ10* as an internal reference gene for comparing data from different PCR runs or cDNA samples (Livak & Schmittgen 2001). To ensure the validity of the $2^{-\Delta\Delta C_T}$ method, twofold serial dilutions of cDNA from unstressed *A. thaliana* and *T. halophila* were used to create standard curves, and the amplification efficiencies of the target and reference genes were shown to be approximately equal (Livak & Schmittgen 2001).

For quantification of unstressed transcript levels, real-time PCR products for each gene were gel purified (QIAEX II Gel Extraction Kit; Qiagen, Valencia, CA, USA) and quantified with a NanoDrop spectrophotometer (ND-1000; NanoDrop Technologies). Tenfold serial dilutions of each PCR product were used to create standard curves for determination of transcript copy number in unstressed plants. At least three values corresponding to the absolute transcript copy number were produced for each sample in three independent experiments. As a loading control, the absolute transcript copy number of *UBQ10* was also calculated and normalized to the highest *UBQ10* level, which was assigned a value of 1. The target gene transcript copy number was then adjusted for loading differences by dividing by normalized *UBQ10* level.

Statistics

Unless otherwise stated, all experiments were repeated three times, and within each experiment, treatments were replicated three times, each replication comprising 10–15 pooled plants. All data were statistically analysed by Fisher's protected least significant difference (LSD) test using SAS statistical software (SAS Institute, Cary, NC, USA).

RESULTS

Growth of *A. thaliana* and *T. halophila* under saline conditions

The *A. thaliana* and *T. halophila* plants were grown in a perlite/soil mixture to allow easy harvesting of roots. This medium was favoured over a hydroponic system because root physiology can differ in hydroponics compared to a soil-based medium (Gibeaut *et al.* 1997). This appears to be

the case for both *A. thaliana* and *T. halophila*, which were able to tolerate higher levels of NaCl when grown in soil than when grown in a hydroponic system (Volkov *et al.* 2004). For salt treatments, NaCl was applied by increasing NaCl concentration in the nutrient solution incrementally to examine adaptive responses to NaCl rather than shock responses.

After one week at the final concentration of each salt level, *A. thaliana* exhibited acute symptoms of stress. The plants showed severe and increasing growth reduction at 100, 250 and 500 mM NaCl, possessing less expanded leaves, many of which showed signs of chlorosis and of purple anthocyanin accumulation (data not shown). *Arabidopsis thaliana* plants grown at NaCl concentrations above 100 mM did not complete their life cycle, while those grown at 750 mM NaCl died after 2 d at the final NaCl concentration. In contrast, *T. halophila* plants exhibited normal growth at 100 and 250 mM NaCl, marginal growth reduction at 500 mM NaCl and only at 750 mM did the plants exhibit signs of severe stress. Figure 1a shows that salt stress-induced growth reduction in *A. thaliana* was reflected in a 25% decrease in leaf number at 100 mM NaCl, while leaf number in *T. halophila* was significantly affected only at and above 500 mM NaCl where it was reduced by 21% compared to control plants. Furthermore, *A. thaliana* exhibited a 58 and 44% reduction in shoot FW and DW, respectively, at 100 mM NaCl, while *T. halophila* showed only a 25% reduction in FW at 250 mM NaCl and a 28% reduction in DW at 500 mM NaCl (Fig. 1b–e). The appearance of purple anthocyanins is often used as a marker of abiotic stress. Anthocyanins could be detected in the shoots of *A. thaliana* plants exposed to 100 mM NaCl, and there was a dramatic rise in anthocyanin content with increasing NaCl concentration (Fig. 1f). In *T. halophila*, however, a significant rise in anthocyanin content could only be detected at 250 mM NaCl. At 500 mM NaCl, *T. halophila* accumulated anthocyanin at a level comparable to *A. thaliana* at 100 mM NaCl. Even at 750 mM NaCl, the anthocyanin content in *T. halophila* was far lower than in *A. thaliana* at 250 mM NaCl.

Real-time PCR analysis of *T. halophila* gene expression

To explore differential expression of salt tolerance orthologues between *A. thaliana* and *T. halophila*, we elected to measure the expression of key genes involved in two responses of plants to salt stress, namely control of Na⁺ transport/accumulation and compatible osmolyte (proline) production. Because *T. halophila* expressed sequence tags (ESTs) exhibit approximately 95% identity with *A. thaliana* cDNA sequences (Bressan *et al.* 2001; Wang *et al.* 2004), we used *A. thaliana* mRNA sequences to design primers for use in real-time PCR measurement of gene expression in both species. Figure 2a shows the PCR products obtained after real-time PCR with *A. thaliana* and *T. halophila* cDNA. Primers designed from the various *A. thaliana* gene sequences were able to amplify a similar-sized PCR product from their respective *T. halophila*

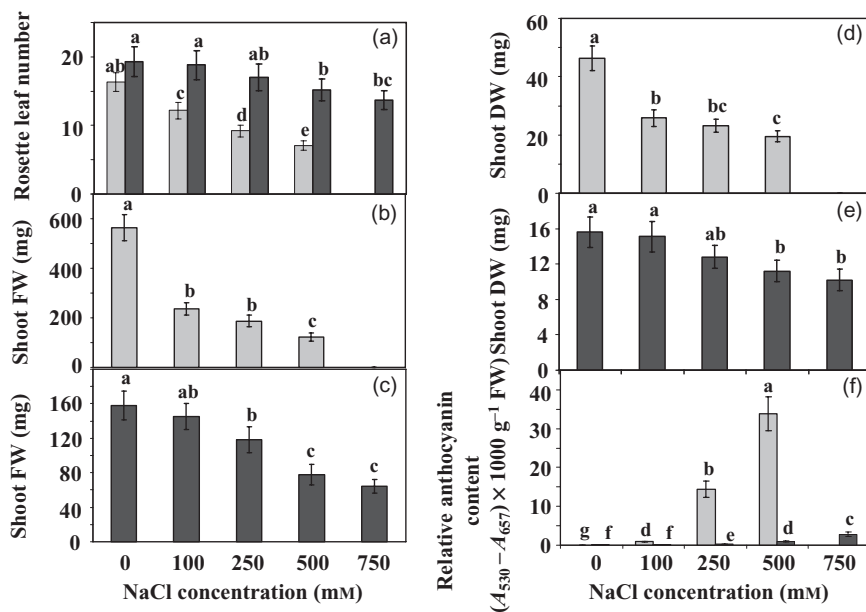


Figure 1. Effect of NaCl concentration on growth parameters and anthocyanin content of *Arabidopsis thaliana* and *Thellungiella halophila*. Ten-day-old *A. thaliana* and 12-day-old *T. halophila* plants were exposed to incremental increases of NaCl concentration. The plants were harvested one week after the final NaCl concentration was reached. Values are mean \pm SD; $n = 3$. Each replicate comprised 10–15 pooled plants. The data are representative of similar results from three independent experiments. At 750 mM NaCl, growth parameters were only measured in *T. halophila*. (a) Rosette leaf number; (b) *A. thaliana* shoot FW; (c) *T. halophila* shoot FW; (d) *A. thaliana* shoot DW; (e) *T. halophila* shoot DW; (f) anthocyanin content. Bars with different letters indicate significant difference at $P < 0.05$ [Fisher's protected least significant difference (LSD) test]. Grey bars, *A. thaliana*; black bars, *T. halophila*. FW, fresh weight; DW, dry weight.

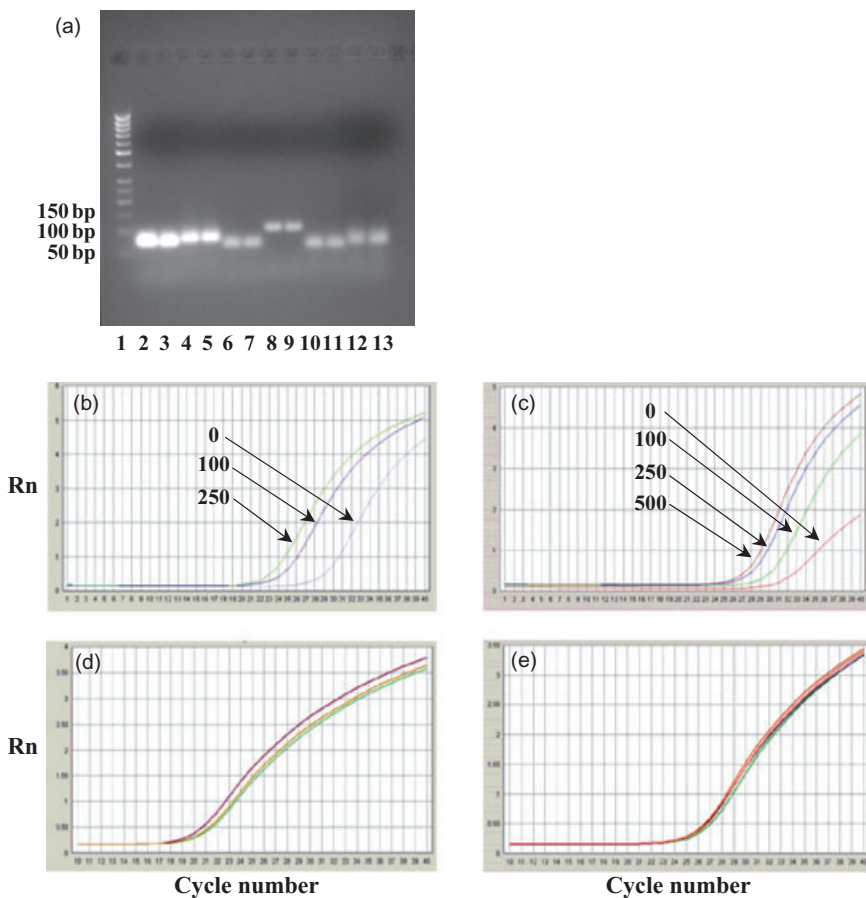


Figure 2. Real-time PCR amplification of *Arabidopsis thaliana* and *Thellungiella halophila* orthologous sequences using primers based on *A. thaliana* gene sequences. Experimental conditions are as described in Fig. 1. (a) Separation of real-time PCR products on a 4% agarose gel revealed a single band of expected size for genes from both plant species. Lane 1, 50 bp size standards; lane 2, *A. thaliana* Δ^1 -pyrroline-5-carboxylate synthetase 1 (*AtP5CS1*); lane 3, *T. halophila* P5CS 1 (*ThP5CS1*); lane 4, *A. thaliana* P5CS 2 (*AtP5CS2*); lane 5, *T. halophila* P5CS 2 (*ThP5CS2*); lane 6, *A. thaliana* ornithine- δ -aminotransferase (*AtOAT*); lane 7, *T. halophila* OAT (*ThOAT*); lane 8, *A. thaliana* proline dehydrogenase (*AtPDH*); lane 9, *T. halophila* PDH (*ThPDH*) (from roots); lane 10, *A. thaliana* ubiquitin 10 (*AtUBQ10*); lane 11, *T. halophila* UBQ10 (*ThUBQ10*); lane 12, *A. thaliana* salt overly sensitive 1 (*AtSOS1*); lane 13, *T. halophila* SOS 1 (*ThSOS1*). Real-time amplification plots of (b) *AtP5CS1*; (c) *ThP5CS1*; (d) *AtUBQ10*; (e) *ThUBQ10* in plants grown under increasing salt levels are presented. The concentration of NaCl (mM) at which each amplification plot was obtained for *AtP5CS1* and *ThP5CS1*, is indicated. The colours of each plot line are arbitrarily assigned by the real-time PCR software and do not represent a particular salt concentration. Rn, normalized reporter.

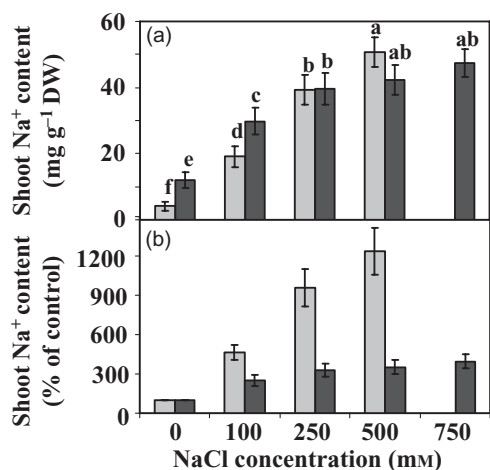


Figure 3. Effect of NaCl on Na⁺ content in shoots of *Arabidopsis thaliana* and *Thellungiella halophila*. Experimental conditions are as described in Fig. 1. Data represent mean \pm SD; $n = 3$. Each replicate comprised 10–15 pooled plants. The data are representative of similar results from three independent experiments. (a) Absolute Na⁺ content on a dry weight (DW) basis. (b) Relative changes in Na⁺ content compared to control treatment. Grey bars, *A. thaliana*; black bars, *T. halophila*. Bars with different letters indicate significant difference at $P < 0.05$ [Fisher's protected least significant difference (LSD) test].

orthologues. Furthermore, dissociation curves indicated that primers designed using *A. thaliana* sequences gave a single PCR product at the product-specific melting temperature using *T. halophila* cDNA as a template (data not shown).

Figure 2b and c show typical real-time PCR amplification plots for *A. thaliana P5CS1* (*AtP5CS1*) and *T. halophila P5CS1* (*ThP5CS1*), respectively, at different concentrations of NaCl. The number of PCR cycles required to reach an Rn threshold of 0.2 decreases as salt concentration rises, indicating increased *P5CS1* expression whereas salt has virtually no effect on the expression of the housekeeping gene *UBQ10*, used as an internal control (Fig. 2d & e). Relative expression levels were normalized to the control (0 mM NaCl) treatment within each species. For direct comparison of unstressed transcript levels between the two species, an absolute quantification of transcript levels was performed by relating the PCR signal to a standard curve produced for each gene from each species.

Comparative analysis of Na⁺ accumulation and expression of *SOS1* in *A. thaliana* and *T. halophila*

Two recent reports have presented evidence that *T. halophila* is able to tightly control Na⁺ accumulation, a characteristic property of halophytes (Inan *et al.* 2004; Volkov *et al.* 2004). Our tissue ion analysis further confirmed this idea. Under non-stress conditions, *T. halophila* possessed about three times as much Na⁺ in the shoots as

A. thaliana (Fig. 3a). This is consistent with the finding that *T. halophila* has a lower osmotic potential in unstressed plants than *A. thaliana* (Inan *et al.* 2004). Although both species accumulated increasing amounts of Na⁺ at higher salt concentrations, *A. thaliana* accumulated Na⁺ to a greater extent than *T. halophila* so that at 250 and 500 mM NaCl, there was no significant difference in Na⁺ content between the two species (Fig. 3a).

This finding is further illustrated in Fig. 3b where Na⁺ content is expressed as a percentage of control Na⁺ levels. Whereas in *A. thaliana*, Na⁺ levels rose dramatically with increasing salt concentration in the nutrient solution compared to control plants, *T. halophila* exhibited a relatively modest rise in Na⁺ accumulation in the 100 mM NaCl treatment with no further significant increase in Na⁺ accumulation in the 250, 500 and 750 mM NaCl treatments. These data were reflected in Na⁺/K⁺ ratios, which were slightly higher in *T. halophila* in unstressed plants and plants exposed to 100 mM NaCl, but were much higher in *A. thaliana* at 250 and 500 mM NaCl (data not shown).

Na⁺ transporters have a critical role in maintaining ion homeostasis during salt stress in *A. thaliana*. One of these, the plasma membrane Na⁺/H⁺ antiporter *A. thaliana SOS1* (*AtSOS1*) (Shi *et al.* 2000), appears to control the loading and retrieval of Na⁺ into and from the xylem stream (Shi *et al.* 2002). Figure 4a shows that in *A. thaliana* shoots, *AtSOS1* expression exhibited little change after 1 week at 100 mM NaCl, but increased by 70% at 250 mM NaCl. On the other hand, *T. halophila SOS1* (*ThSOS1*) expression was already induced twofold at 100 mM NaCl with a further significant rise in expression at 500 mM NaCl (Fig. 4b). In the roots of both species, *SOS1* transcript only slightly increased in response to salt (Fig. 4c & d).

A comparison of *SOS1* transcript copy number in unstressed plants revealed that the two species had similar levels of *SOS1* transcript in their shoots, while *T. halophila* possessed over threefold more *SOS1* transcript in its roots than *A. thaliana* (Table 1). Furthermore, while *A. thaliana* had about twofold more *AtSOS1* transcript in roots than in shoots, *T. halophila* possessed approximately sevenfold more *ThSOS1* transcript in roots than in shoots.

These comparative *SOS1* expression results are consistent with our tissue Na⁺ ion results, which show that *T. halophila* accumulates salt to a lower extent under stress than *A. thaliana* (Fig. 3b). However, because *SOS1* appears to function in xylem loading and retrieval of Na⁺, we might expect *T. halophila* to exhibit lower stress-induced accumulation of Na⁺ in the xylem sap than *A. thaliana*. Therefore, to further link differences in *SOS1* expression to beneficial effects on *T. halophila* salt tolerance, we measured the Na⁺ concentration in the xylem sap of *A. thaliana* and *T. halophila* plants either grown under control conditions or subjected to 24 h of 100 mM salt stress. Table 2 shows that under control conditions, *T. halophila* possessed higher Na⁺ concentrations in xylem sap than *A. thaliana*. However, *A. thaliana* xylem sap Na⁺ concentration rose almost 240% under salt stress compared to control plants. In contrast,

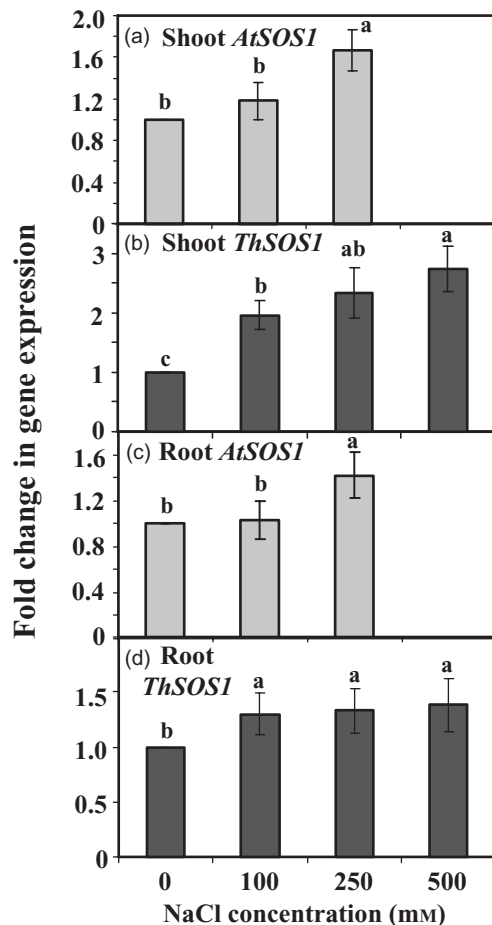


Figure 4. Effect of NaCl on the expression of *salt overly sensitive 1 (SOS1)* in shoots and roots of *Arabidopsis thaliana* and *Thellungiella halophila*. Experimental conditions are as described in Fig. 1. Relative transcript levels were determined by real-time PCR according to the $2^{-\Delta\Delta C_T}$ method using *ubiquitin 10 (UBQ10)* as an internal control (Livak & Schmittgen 2001). The gene expression at each salt level was normalized to the unstressed expression level, which was assigned a value of 1. At 500 mM NaCl, expression was only measured in *T. halophila*. Data represent the average of three independent experiments \pm SD. (a) Shoot *A. thaliana* salt overly sensitive 1 (*AtSOS1*); (b) Shoot *T. halophila* *SOS1* (*ThSOS1*); (c) Root *AtSOS1*; (d) Root *ThSOS1*. Bars with different letters indicate significant difference at $P < 0.05$ [Fisher's protected least significant difference (LSD) test]. Grey bars, *A. thaliana*; black bars, *T. halophila*. *AtSOS1*, *A. thaliana* *SOS 1*; *ThSOS1*, *T. halophila* *SOS 1*.

T. halophila xylem sap Na^+ rose only 87% under salt stress compared to control conditions.

Comparative analysis of proline accumulation and expression of genes encoding proline biosynthetic and catabolic enzymes in *A. thaliana* and *T. halophila*

The second set of genes linked with stress tolerance that we chose to analyse were those involved in the accumulation of proline. Proline is a major compatible osmolyte that

accumulates in *T. halophila* under salt stress (Inan *et al.* 2004). Furthermore, proline levels are higher in unstressed *T. halophila* plants grown in hydroponics than in unstressed *A. thaliana* plants, suggesting that stress tolerance mechanisms in *T. halophila* are active even in the absence of stress (Taji *et al.* 2004). It is unknown, however, whether salt stress induces different levels of proline in *A. thaliana* and *T. halophila* and whether there are any organ-specific differences in proline accumulation. We therefore compared the effect of salt stress on proline levels in our soil-grown *A. thaliana* and *T. halophila* plants in both shoots and roots. In the shoots of unstressed plants, *A. thaliana* possessed very low levels of proline whereas *T. halophila* accumulated about 19-fold more proline (Fig. 5a), in good agreement with data from hydroponic-grown plants that exhibited

Table 1. Transcript copy numbers of stress-related genes in shoots and roots of *Arabidopsis thaliana* and *Thellungiella halophila* under unstressed control conditions

Gene	<i>A. thaliana</i>		<i>T. halophila</i>	
	Shoot	Root	Shoot	Root
<i>SOS1</i>	42 \pm 8.7 ^c	100 \pm 22 ^b	46 \pm 12 ^c	325 \pm 48 ^a
<i>P5CS1</i>	127 \pm 28 ^a	11 \pm 1.5 ^c	27 \pm 6.1 ^b	25 \pm 5.5 ^b
<i>P5CS2</i>	59 \pm 11 ^c	290 \pm 56 ^a	135 \pm 31 ^b	295 \pm 38 ^a
<i>PDH</i>	2.9 \pm 0.6 ^c	96 \pm 12 ^a	ND	21 \pm 2.9 ^b

Quantification of transcript copy number was performed by relating the real-time PCR signal for each gene to a standard curve. The target gene transcript copy number was then adjusted for loading differences by dividing by normalized *ubiquitin 10 (UBQ10)* level. The table represents the average results from three independent experiments \pm SD. Transcript copy number values for each gene with different letters (a–d) are significantly different at $P < 0.05$ [Fisher's protected least significant difference (LSD) test]. *SOS1*, *salt overly sensitive 1*; *P5CS1*, Δ^1 -pyrroline-5-carboxylate synthetase 1; *P5CS2*, Δ^1 -pyrroline-5-carboxylate synthetase 2; *PDH*, *proline dehydrogenase*; ND, not detectable.

Table 2. Effect of salinity on Na^+ concentration in the xylem sap of *Arabidopsis thaliana* and *Thellungiella halophila*

Species	NaCl in nutrient solution (mM)	Xylem sap Na^+ concentration (mM)
<i>A. thaliana</i>	0	4.8 \pm 0.7 ^d
	100	16.2 \pm 0.7 ^b
<i>T. halophila</i>	0	12.2 \pm 0.8 ^c
	100	22.8 \pm 0.8 ^a

Twenty-five-day-old *A. thaliana* and 28-day-old *T. halophila* plants were irrigated with one-third strength Hoagland's solution with or without 100 mM NaCl. Twenty-four hours later, the xylem sap was collected according to Shi *et al.* (2002). Each replicate comprised the pooled sap of three to six plants. Xylem Na^+ values are mean \pm SD, $n = 3$ –5. Data are representative of two independent experiments. Xylem sap concentration values with different letters (a–d) are significantly different at $P < 0.05$ [Fisher's protected least significant difference (LSD) test].

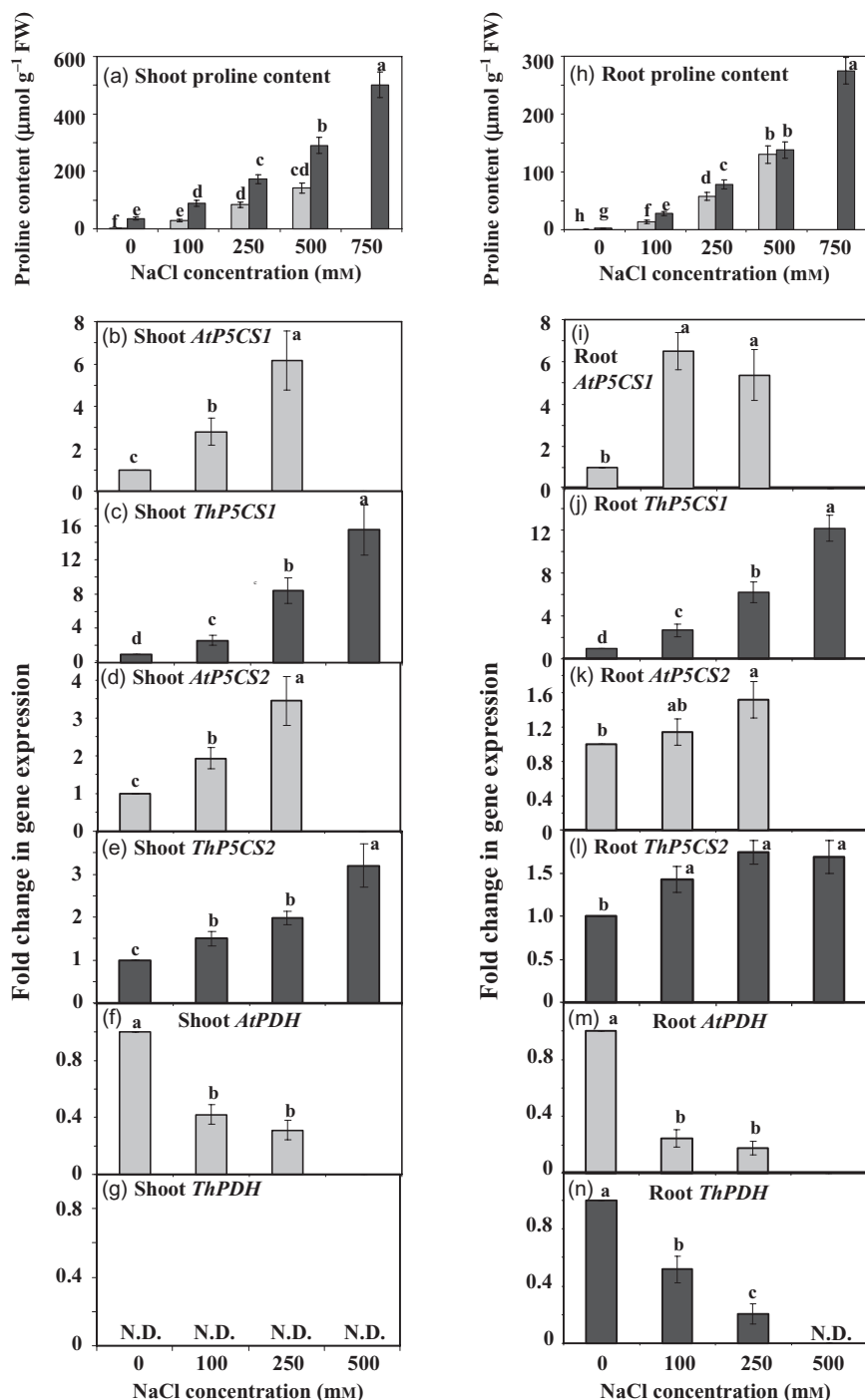


Figure 5. Effect of NaCl on the accumulation of proline and on the expression of genes involved in proline biosynthesis and catabolism, in shoots and roots of *Arabidopsis thaliana* and *Thellungiella halophila*. Experimental conditions are as described in Fig. 1. For proline content, data represent the mean \pm SD; $n = 3$. Each replicate comprised 10–15 pooled plants. The data are representative of similar results from three independent experiments. Relative transcript levels were determined by real-time PCR according to the $2^{-\Delta\Delta C_T}$ method using *ubiquitin 10 (UBQ10)* as an internal control (Livak & Schmittgen 2001). The gene expression at each salt level was normalized to the unstressed expression level, which was assigned a value of 1. At 500 mM NaCl, expression was only measured in *T. halophila*. Data represent the average of three independent experiments \pm SD. (a) Shoot proline content; (b) Shoot *A. thaliana* Δ^1 -pyrroline-5-carboxylate synthetase 1 (*AtP5CS1*) expression; (c) Shoot *T. halophila* *P5CS1* (*ThP5CS1*) expression; (d) Shoot *AtP5CS2* expression; (e) Shoot *ThP5CS2* expression; (f) Shoot *A. thaliana* proline dehydrogenase (*AtPDH*) expression; (g) Shoot *ThPDH* expression; (h) Root proline content; (i) Root *AtP5CS1* expression; (j) Root *ThP5CS1* expression; (k) Root *AtP5CS2* expression; (l) Root *ThP5CS2* expression; (m) Root *AtPDH* expression; (n) Root *ThPDH* expression. Bars with different letters indicate significant difference at $P < 0.05$ [Fisher's protected least significant difference (LSD) test]. Grey bars, *A. thaliana*; black bars, *T. halophila*. FW, fresh weight ND, not detectable.

approximately 12-fold more proline in *T. halophila* (Taji *et al.* 2004). Proline accumulated to higher levels in the shoots of both species during salt stress with rising NaCl concentrations inducing increased proline accumulation (Fig. 5a). However, at each NaCl concentration, *T. halophila* exhibited two- to threefold more proline per unit FW than observed in *A. thaliana* shoots.

In the roots of both species, proline accumulation showed a similar induction by salt stress as that observed in shoots (Fig. 5h). However, there were also some sig-

nificant differences. Firstly, absolute levels of proline at each NaCl level were lower in roots than in shoots, suggesting a gradient of osmotic adjustment from root to shoot. Secondly, the unstressed level of proline was only sixfold higher in *T. halophila* roots than in *A. thaliana* roots compared to a 19-fold difference in shoots (Fig. 5a). Thirdly, whereas in shoots the difference in proline content between *A. thaliana* and *T. halophila* at each NaCl level remained fairly constant; in roots, this difference gradually disappeared. Thus, root proline

levels were virtually identical between the two species at 500 mM NaCl.

Stress-induced proline accumulation in plants has been shown to be the result of both an increase in proline biosynthesis and repression of proline catabolism (Delauney & Verma 1993; Kiyosue *et al.* 1996; Peng, Lu & Verma 1996; Verbruggen *et al.* 1996; Nakashima *et al.* 1998). Two proline biosynthetic pathways exist in higher plants (Delauney & Verma 1993; Kavi-Kishor *et al.* 2005). Δ^1 -pyrroline-5-carboxylate synthetase (P5CS) is the key enzyme of the pathway from glutamate to proline, and two isoforms are encoded by *AtP5CS1* and *A. thaliana P5CS 2 (AtP5CS2)* (Strizhov *et al.* 1997). The key enzyme of the alternative proline biosynthetic pathway via ornithine is OAT. Conversely, the first step in proline catabolism is catalysed by PDH encoded by *A. thaliana PDH (AtPDH)* (Kiyosue *et al.* 1996). To test whether the differences in proline accumulation observed between *A. thaliana* and *T. halophila* can be attributed to differences in gene regulation, we measured the expression of genes encoding proline biosynthesis and degradation enzymes. The measurement of transcript copy numbers in the shoots of unstressed plants revealed approximately twofold more *AtP5CS1* than *AtP5CS2* mRNA but fivefold more *T. halophila P5CS 2 (ThP5CS2)* than *ThP5CS1* transcript (Table 1), suggesting that in *T. halophila* shoots, *ThP5CS2* is the dominant P5CS isoform. *Arabidopsis thaliana* shoots contained more *P5CS1* transcript than *T. halophila* but about half the level of *P5CS2* transcript. No species-specific difference in shoot OAT transcript copy number was observed (data not shown). Both species showed similar salt induction of expression of the two *P5CS* genes in shoots, with *P5CS1* expression exhibiting a greater response to NaCl than *P5CS2* (Fig. 5b–e). Salt stress caused little change in either *A. thaliana OAT (AtOAT)* or *T. halophila OAT (ThOAT)* expression with a significant (twofold) increase only observed at 250 mM NaCl (data not shown).

The most striking finding was that *T. halophila PDH (ThPDH)* transcript was undetectable in shoots in all treatments (Table 1 and Fig. 5g). In contrast, *AtPDH* transcript was detected at all NaCl levels, but exhibited sharp repression of expression under salt stress (Fig. 5f).

As noted previously, *A. thaliana* and *T. halophila* roots also accumulated proline in response to salt stress, although there was less of a difference in proline levels between the two species. *P5CS2* was the major *P5CS* transcript in unstressed roots of both species and was present in significantly higher amounts in roots than in shoots (Table 1). *AtP5CS1* transcript was present in lower amounts in *A. thaliana* roots than in shoots, while there was little difference in *ThP5CS1* mRNA levels between the two organs of *T. halophila*. No differences were observed between *AtOAT* and *ThOAT* transcript copy numbers in unstressed roots (data not shown). Under salt stress, *P5CS1* expression exhibited a much higher relative increase compared to controls than *P5CS2* in the roots of both species (Fig. 5i–l). Moreover, at 250 mM there was no significant difference in *AtP5CS1* expression levels compared to 100 mM, while

ThP5CS1 expression rose with increasing NaCl concentration. No significant salt stress-mediated increase in root OAT expression was observed in either species (data not shown). Most notable were unstressed levels of root PDH transcript (Table 1). Here, *ThPDH* transcript was detectable at a level approximately 4.5-fold less than that of *A. thaliana* root *AtPDH*. The fact that *ThPDH* was present in *T. halophila* roots is consistent with our results in unstressed roots showing only sixfold higher proline levels in *T. halophila* compared to *A. thaliana* while in shoots, where *ThPDH* was undetectable, a 19-fold difference was observed. Under salt stress, PDH expression in the roots of both species dropped sharply (Fig. 5m & n). At 500 mM NaCl, *ThPDH* transcript was no longer detectable.

The finding that expression of the *T. halophila* ortholog of *AtPDH* is undetectable in *T. halophila* shoots and is reduced in roots compared to *A. thaliana* could account for the increased proline levels observed in *T. halophila*. However, it is possible that *T. halophila*, which has a considerably larger genome than *A. thaliana* (Bressan *et al.* 2001), possesses one or more additional isoforms of PDH. Such a gene might not have been amplified with primers designed for *AtPDH*. To examine this possibility and to test whether the absence or reduction of *ThPDH* expression leads to reduced PDH enzyme activity, we measured PDH activity in *A. thaliana* and *T. halophila* shoots and roots.

In shoots, the PDH enzyme activity was detected in both species indicating that at least one other PDH isoform is likely active in *T. halophila* (Fig. 6a). Nevertheless, *T. halophila* PDH activity was consistently about half that of *A. thaliana* in both unstressed and stressed plants, and followed a similar pattern of suppression with increasing salt level as was observed when PDH gene expression was analysed (Fig. 5m & n). Similar results were obtained in roots except that the overall levels of PDH activity were about fourfold higher than in shoots (Fig. 6b).

It has been previously shown that *A. thaliana* seedlings containing either a transferred DNA (T-DNA) insertion in *AtPDH* or an antisense copy of the gene, exhibit hyperaccumulation of proline and reduced growth when supplied with exogenous proline (Mani *et al.* 2002; Nanjo *et al.* 2003). To further confirm, therefore, that *T. halophila* has a reduced capacity for proline catabolism, *A. thaliana* and *T. halophila* seedlings were transferred to MS plates (Murashige & Skoog 1962) containing increasing levels of proline. *Arabidopsis thaliana* plants showed similar growth under both control and 10 mM proline-rich media (Fig. 7a & b). On the other hand, proline-grown *T. halophila* seedlings showed severe growth reduction as well as pale chlorotic cotyledons (Fig. 7d) compared to control seedlings (Fig. 7c). To quantify the response of *A. thaliana* and *T. halophila* to proline-rich media, the increase in seedling root elongation after transfer to a range of proline concentrations was measured (Fig. 7e). Exogenous proline had no effect on *A. thaliana* root elongation at all proline concentrations employed. *Thellungiella halophila* root elongation, however, exhibited increasing root growth retardation as proline concentration was increased. Growth retardation

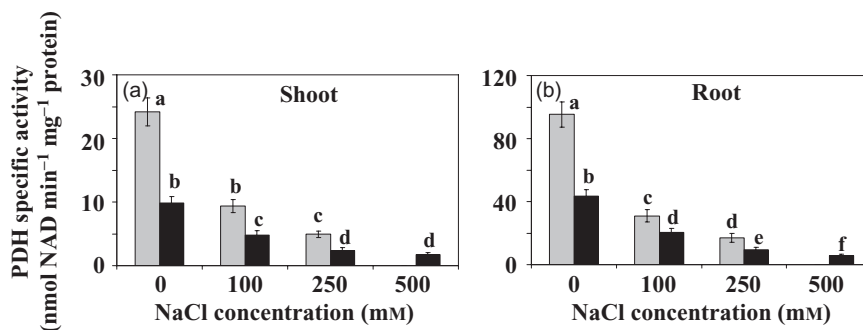


Figure 6. Effect of NaCl on proline dehydrogenase (PDH) enzyme activity in shoots and roots of *Arabidopsis thaliana* and *Thellungiella halophila*. Experimental conditions are as described in Fig. 1. At 500 mM NaCl, activity was only measured in *T. halophila*. Data represent the mean \pm SD; $n = 3$. Each replicate comprised 10–15 pooled plants. The data are representative of similar results from three independent experiments. (a) Shoot proline dehydrogenase (PDH) activity; (b) Root PDH activity. Bars with different letters indicate significant difference at $P < 0.05$ [Fisher's protected least significant difference (LSD) test]. Grey bars, *A. thaliana*; black bars, *T. halophila*.

was specific to proline because the growth of seedlings on alanine-enriched media (alanine belongs to the same family of amino acids as proline) had no effect on root elongation in either species (Fig. 7f). This result supports the notion that the sensitivity of *T. halophila* to exogenous proline is because of the decreased PDH activity rather than general sensitivity to exogenous supply of amino acids.

DISCUSSION

Salt tolerance strategies of *T. halophila*

The characterization of *T. halophila* as an ARMS by Bresnan *et al.* (2001) and Inan *et al.* (2004) has brought about the possibility of rigorous investigation of the molecular, biochemical and physiological bases of the extreme salt tolerance of halophytes. As clearly shown by the data in the present study (Fig. 1) and in other recent reports (Inan *et al.* 2004; Taji *et al.* 2004; Volkov *et al.* 2004), *T. halophila* was able to tolerate very high (500 mM) levels of NaCl salinity. On the other hand, *A. thaliana* plants exhibited severe symptoms of stress at 100 mM NaCl, and at concentrations above this, failed to complete their life cycle. The ability of *T. halophila* to tolerate such high salinity levels is, at least partly, based on mechanisms typical of halophytes: controlled accumulation of NaCl (Fig. 3 and Table 2; Inan *et al.* 2004; Vera-Estrella *et al.* 2005), production of high levels of the osmoprotectant proline (Fig. 5a & h; Inan *et al.* 2004) and various morphological adaptations such as succulent leaves and development of a second endodermis that may aid in restricting movement of salt from the root to the shoot (Inan *et al.* 2004). However, many of these salt tolerance mechanisms and associated genes are also found in *A. thaliana* (Zhu 2000, 2001; Xiong & Zhu 2002a; Shinozaki, Shinozaki-Yamaguchi & Seki 2003), and it is now hypothesized that subtle differences in gene regulation might partly explain the differences in the ability of glyco-phytes and halophytes to tolerate extreme levels of salinity (Hasegawa *et al.* 2000; Zhu 2000, 2001). The recent analysis of *A. thaliana* and *T. halophila* transcript profiles showing

that 60% of stress-regulated genes were differentially regulated between the two species further strengthens this idea (Gong *et al.* 2005).

Differential expression of *AtSOS1* and *ThSOS1* and the control of Na⁺ accumulation

Our results comparing expression of *SOS1* in *A. thaliana* and *T. halophila* revealed that shoot *SOS1* levels differ little under control conditions but are more strongly induced by salt in *T. halophila*, whereas root *SOS1* levels are constitutively higher in *T. halophila* but show little inter-species differences in their response to salt (Fig. 4 and Table 1). This constitutive expression of root *ThSOS1* could be a contributing factor to the higher shoot Na⁺ content in unstressed *T. halophila* plants (Fig. 3a), which is a feature of many halophytes (Tester & Davenport 2003). Under mild stress, *AtSOS1* is thought to function in loading Na⁺ into the xylem (Shi *et al.* 2002), and it is conceivable that *SOS1* plays a similar role in unstressed *T. halophila*. This notion is supported by our analysis of xylem sap showing higher Na⁺ concentrations in unstressed *T. halophila* compared to *A. thaliana*. Furthermore, the higher unstressed levels of Na⁺ correlate with a lower leaf osmotic potential of unstressed *T. halophila* compared to *A. thaliana* (Inan *et al.* 2004). Conversely, under severe salt stress, *AtSOS1* appears to be responsible for retrieving Na⁺ from the xylem stream (Shi *et al.* 2002). Moreover, *A. thaliana* plants that overexpress *SOS1* are more tolerant to salt because of this Na⁺ retrieval (Shi *et al.* 2003). Thus, in *T. halophila*, the salt-mediated induction of shoot *ThSOS1* expression coupled with high basal root *ThSOS1* expression is likely to be a crucial factor in tightly controlling the extent of shoot Na⁺ accumulation. Consistent with this idea, the increase in Na⁺ concentration of *T. halophila* xylem sap under salinity compared with control xylem Na⁺ levels was much lower than in *A. thaliana* (Table 2).

Taji *et al.* (2004) also observed higher unstressed levels of *SOS1* transcript in *T. halophila* compared to *A. thaliana*, by both microarray analysis and Northern blot. However,

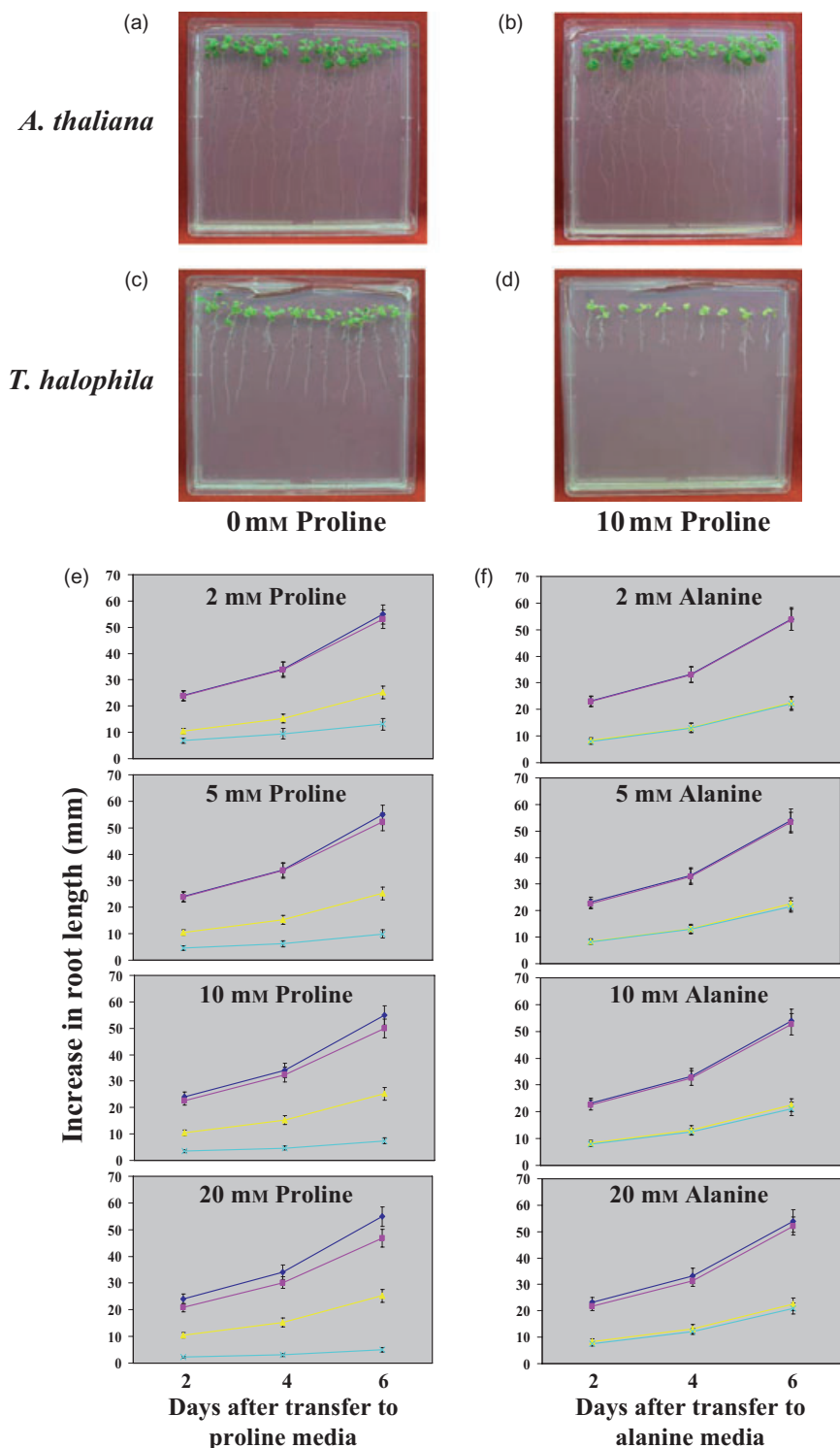


Figure 7. Effect of exogenous proline supply on the growth of *Arabidopsis thaliana* and *Thellungiella halophila* seedlings. Four-day-old *A. thaliana* seedlings and 6-day-old *T. halophila* seedlings were transferred to Murashige and Skoog (MS) plates with or without the addition of 10 mM L-proline. (a–d) *Arabidopsis thaliana* and *T. halophila* seedlings 6 d after transfer to control and 10 mM proline plates.

(e) Increases in root length were measured every 2 d after transfer to control and proline plates. (f) Increases in root length were measured every 2 d after transfer to control and alanine plates. Data are presented as mean \pm SD; $n = 3$. Each replicate comprised the average increase in root length of 10–12 seedlings. The data are representative of similar results from two independent experiments. Fisher's protected least significant difference (LSD) test showed a significant difference ($P < 0.05$) between *A. thaliana* and *T. halophila* root lengths. No significant effects of proline were found on *A. thaliana* root elongation, while proline significantly affected ($P < 0.05$) *T. halophila* root elongation at all proline concentrations and time points except for 2 d on 2 mM proline. No significant effects of alanine were found for either species. Dark blue line, *A. thaliana* control; Purple line, *A. thaliana* proline-grown; Yellow line, *T. halophila* control; Pale blue line, *T. halophila* proline-grown.

both Taji *et al.* (2004) and Gong *et al.* (2005) were unable to show induction of *ThSOS1* by salt treatment. On the other hand, Vera-Estrella *et al.* (2005) observed salt-mediated increases in SOS1 protein levels in *T. halophila*.

One possibility is that the plants used in the microarray studies (Taji *et al.* 2004; Gong *et al.* 2005) were subjected to short salt shocks of between 2 and 24 h. In contrast, salinity

treatments in the present study were applied incrementally over a period of days until the final NaCl concentration was reached, thereby minimizing shock responses and allowing plants to adapt to the saline conditions (Tester & Davenport 2003). Furthermore, all analyses were conducted a week after the final NaCl concentration was attained. Vera-Estrella *et al.* (2005), although employing salt shock, carried

out their analyses two weeks after commencement of NaCl treatments. Considering that *T. halophila* possesses a root morphology presumably designed to minimize the flow of ions, exhibits low rates of transpiration (Inan *et al.* 2004) and already contains higher unstressed salt levels than *A. thaliana*, it is possible that short salt shock treatments do not elicit induction of *ThSOS1* expression.

***Thellungiella halophila* exhibits a reduced capacity for proline catabolism**

A dramatic example of differential regulation of gene expression between *A. thaliana* and *T. halophila* is the observation that expression of *PDH* in *T. halophila* shoots is completely repressed (Table 1 and Fig. 5g). The fact that *ThPDH* expression was detected in *T. halophila* roots (Table 1 and Fig. 5n) suggests that *ThPDH* transcript was undetectable in shoots because of repression of its expression rather than non-annealing of primers in the real-time PCR reaction. Although *PDH* enzyme activity was detected in shoots suggesting the presence of another gene with *PDH* function, this activity was consistently half that of *A. thaliana* at each salt level (Fig. 6). A reduced capacity for proline catabolism was further supported by results showing hypersensitivity of *T. halophila* seedlings to exogenous supply of proline (Fig. 7). Changes in *PDH* expression can have large effects on free proline levels. Hence, in transgenic *A. thaliana* plants expressing an antisense *AtPDH* gene, free proline levels were enhanced by 25% on an FW basis (Mani *et al.* 2002). Thus, repression of *ThPDH* expression could effectively contribute to the higher levels of proline observed in unstressed *T. halophila* shoots compared to *A. thaliana* (Fig. 5a). The link between repressed *ThPDH* expression and increased proline levels is further supported by our analysis of organ-specific *PDH* gene expression, *PDH* enzyme activity and proline levels (Figs 5 & 6). *Thellungiella halophila* roots exhibit: (1) reduced absolute proline levels compared to shoots; and (2) a much smaller difference in proline levels between *A. thaliana* and *T. halophila* than in shoots. These features of root proline levels fit in well with the observations that *ThPDH* is expressed in *T. halophila* roots but not in shoots, and that roots exhibit higher overall root *PDH* enzyme activity compared to shoots. Thus, the *PDH* gene expression and enzyme activity results coupled with the fact that basic expression and regulation of the *P5CS* isoforms are rather similar in the two species, lend credence to the assertion that differential regulation of *PDH* expression between *A. thaliana* and *T. halophila* is the major determinant of high proline levels in the halophyte.

The question remains, however, as to the degree to which the higher proline levels observed in *T. halophila* contribute to its tolerance to extreme levels of salt. Several reports suggest that increased proline levels lead to improved stress tolerance. For instance, over-expression of mothbean *P5CS* in transgenic tobacco led to enhanced tolerance to salinity (Kavi-Kishor *et al.* 1995). Similarly, transgenic tobacco

harbouring a mutated *P5CS* that no longer responded to negative feedback regulation by proline, was also more tolerant to salt (Hong *et al.* 2000). More pertinent to the present study, transgenic *A. thaliana* containing an antisense copy of *AtPDH* led to higher proline levels and increased salt and freezing tolerance compared to wild-type plants (Nanjo *et al.* 1999). On the other hand, the antisense *AtPDH* *A. thaliana* plants generated by Mani *et al.* (2002) showed no such enhancement of stress tolerance. Carrot cell lines that exhibited a sixfold increase in proline levels also showed no improvement in stress tolerance (Maggio *et al.* 1997). There are thus some inconsistencies in experimental results that have attempted to link increased proline levels with enhanced stress tolerance. Nevertheless, our results showing salt induction of *T. halophila* *P5CS* (*ThP5CS*) expression, repression of *ThPDH* expression (in roots) together with the clear differences in control of *AtPDH* and *ThPDH* expression lead us to suggest that proline, at least partly, contributes to the enhanced salt tolerance of *T. halophila*. Higher unstressed levels and salt-induced increases in soluble sugar content have also been observed in *T. halophila* compared to *A. thaliana* (Inan *et al.* 2004; Gong *et al.* 2005), and recent EST analyses of two salt-stressed *T. halophila* ecotypes have identified genes related to sugar synthesis, sugar transport and glycine betaine synthesis (Wang *et al.* 2004; Wong *et al.* 2005). It is likely therefore that higher levels of several compatible osmolytes/osmoprotectants play a role in *T. halophila* salt tolerance, and we are currently producing *T. halophila* plants that overexpress *ThPDH* to examine the relative contribution of proline.

An evolutionary perspective

Inan *et al.* (2004) posited three hypotheses regarding the development of mechanisms in plants that can naturally tolerate extreme environmental conditions: (1) high tolerance is caused by divergent promoter structures; (2) the evolution of more active forms of gene products can confer tolerance; and (3) tolerance is conferred by the presence of unique stress response genes.

Many evolutionary biologists consider that evolution often proceeds by changes in the spatial and temporal patterns of gene expression (Doebley & Lukens 1998). Protein function is apt to be conserved over long evolutionary periods, while changes in promoter structure could generate diverse patterns of gene expression, thereby producing novel phenotypes. Furthermore, plants are able to tolerate drastic sequence changes in *cis*-acting regulatory regions such as insertions and rearrangements (Wessler, Bureau & White 1995). Our results showing differential regulation of *SOS1* and *PDH* expression between closely related glyco-phytic and halophytic species support the idea that evolutionary changes in promoter structure contribute to the development of salt tolerance mechanisms in *T. halophila*. On the other hand, we cannot rule out the possibility that the observed expression patterns are the result of differences in RNA stability, and this is particularly pertinent in

the case of *SOS1* where *AtSOS1* transcript is less stable in the absence of salt stress (Shi *et al.* 2003). Furthermore, the predominance of *P5CS2* transcript in *T. halophila* shoots (Table 1) may indicate that plant-specific differences at the level of protein structure (hypothesis 2) are also relevant. In addition, the finding that some *T. halophila* ESTs represented genes not found in *A. thaliana* (Wong *et al.* 2005) suggests that *T. halophila* may also contain unique stress tolerance genes (hypothesis 3).

It will now be of interest to compare the architecture and regulatory elements of promoters regulating the expression of orthologous *A. thaliana* and *T. halophila* genes whose differential expression patterns contribute to the halophytic characteristics of *T. halophila*.

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REFERENCES

- Apel K. & Hirt H. (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annual Review of Plant Biology* **55**, 373–399.
- Apse M.P., Aharon G.S., Snedden W.A. & Blumwald E. (1999) Salt tolerance conferred by overexpression of a vacuolar Na⁺/H⁺ antiporter in *Arabidopsis*. *Science* **285**, 1256–1258.
- Asada K. (1997) The role of ascorbate peroxidase and monohydroascorbate reductase in H₂O₂ scavenging in plants. In *Oxidative Stress and the Molecular Biology of Antioxidant Defenses* (ed. J.G. Scandalios), pp. 353–373. Cold Spring Harbor Laboratory Press, New York, USA.
- Bartley G.E. & Scolnik P.A. (1995) Plant carotenoids: pigments for photoprotection, visual attraction and human health. *Plant Cell* **7**, 1027–1038.
- Bates L.S., Waldren R.P. & Teare I.D. (1973) Rapid determination of free proline for water-stress studies. *Plant and Soil* **39**, 205–207.
- Bradford M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Annals of Biochemistry* **72**, 248–254.
- Bray E.A., Bailey-Serres J. & Weretilnyk E. (2000) Responses to abiotic stresses. In *Biochemistry and Molecular Biology of Plants* (eds B.B. Buchanan, W. Gruissem & R.L. Jones), pp. 1158–1203. American Society of Plant Physiologists, Rockville, MD, USA.
- Bressan R.A., Zhang C., Zhang H., Hasegawa P.M., Bohnert H.J. & Zhu J.-K. (2001) Learning from the *Arabidopsis* experience. The next gene search paradigm. *Plant Physiology* **127**, 1354–1360.
- Burdon R.H., O’Kane D., Fadzilla N., Gill V., Boyd P.A. & Finch R.R. (1996) Oxidative stress and responses in *Arabidopsis thaliana* and *Oryza sativa* subjected to chilling and salinity stress. *Biochemical Society Transactions* **24**, 469–472.
- Chalker-Scott L. (1999) Environmental significance of anthocyanins in plant stress responses. *Photochemistry and Photobiology* **70**, 1–9.
- Chaves M.M., Pereira J.S., Maroco J., Rodrigues M.L., Ricardo C.P.P., Osorio M.L., Carvalho I., Faria T. & Pinheiro C. (2002) How plants cope with water stress in the field. Photosynthesis and growth. *Annals of Botany* **89**, 907–916.
- Chen W., Provart N.J., Glazebrook J., *et al.* (2002) Expression profile matrix of *Arabidopsis* transcription factor genes suggests their putative functions in response to environmental stresses. *Plant Cell* **14**, 559–574.
- Delauney A.J. & Verma D.P.S. (1993) Proline biosynthesis and osmoregulation in plants. *Plant Journal* **4**, 215–223.
- Dixon R.A. & Paiva N.L. (1995) Stress-induced phenylpropanoid metabolism. *Plant Cell* **7**, 1085–1097.
- Doebley J. & Lukens L. (1998) Transcriptional regulators and the evolution of plant form. *Plant Cell* **10**, 1075–1082.
- Eimer M. (2004) Transgenic drought and salt tolerant plants. *Genetic Engineering Newsletter, Special Issue* **15**, 1–14.
- Gaxiola R., Li J., Undurraga S., Dang L.M., Allen G.J., Alper S.L. & Fink G.R. (2001) Drought- and salt-tolerant plants result from overexpression of the AVP1 H⁺ pump. *Proceedings of the National Academy of Sciences of the USA* **99**, 11444–11449.
- Gibeaut D.M., Hulett J., Cramer G.R. & Seemann J.R. (1997) Maximal biomass of *Arabidopsis thaliana* using a simple, low-maintenance hydroponic method and favorable environmental conditions. *Plant Physiology* **115**, 317–319.
- Gong Q., Li P., Ma S., Rupassara S.I. & Bohnert H.J. (2005) Salinity stress adaptation competence in the extremophile *Thellungiella halophila* in comparison with its relative *Arabidopsis thaliana*. *Plant Journal* **44**, 826–839.
- Hasegawa P.M., Bressan R.A., Zhu J.-K. & Bohnert H.J. (2000) Plant cellular and molecular responses to high salinity. *Annual Review of Plant Physiology and Plant Molecular Biology* **51**, 463–499.
- Hoagland D.F. & Arnon D.I. (1950) The water culture for growing plants without soil. *Berkeley, California Agriculture Experimental Station Circular* **347**, 39.
- Hong Z., Lakkineni K., Zhang Z. & Verma D.P.S. (2000) Removal of feedback inhibition of 1-pyrroline-5-carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress. *Plant Physiology* **122**, 1129–1136.
- Inan G., Zhang Q., Li P., *et al.* (2004) Salt cress. A halophyte and cryophyte *Arabidopsis* relative model system and its applicability to molecular genetic analyses of growth and development of extremophiles. *Plant Physiology* **135**, 1718–1737.
- Kavi-Kishor P.B., Hong Z., Miao G.H., Hu C.-A.A. & Verma D.P.S. (1995) Overexpression of Δ¹-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. *Plant Physiology* **108**, 1387–1394.
- Kavi-Kishor P.B., Sangam S., Amrutha R.N., Sri Laxmi P., Naidu K.R., Rao K.R.S.S., Rao S., Reddy K.J., Theriappan P. & Sreenivasulu N. (2005) Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: its implications in plant growth and abiotic stress tolerance. *Current Science* **88**, 424–438.
- Kiyosue T., Yoshida Y., Yamaguchi-Shinozaki K. & Shinozaki K. (1996) A nuclear gene encoding mitochondrial proline dehydrogenase, an enzyme involved in proline metabolism, is upregu-

- lated by proline but downregulated by dehydration in *Arabidopsis*. *Plant Cell* **8**, 1323–1335.
- Kreps J.A., Wu Y., Chang H.-S., Zhu T., Wang X. & Harper J.F. (2002) Transcriptome changes for *Arabidopsis* in response to salt, osmotic and cold stress. *Plant Physiology* **130**, 2129–2141.
- Lawlor D. (1995) The effects of water deficit on photosynthesis. In *Environment and Plant Metabolism* (ed. N. Smirnov), pp. 129–160. Bios Scientific Publishers, Oxford, UK.
- Livak K.J. & Schmittgen T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* **25**, 402–408.
- Lutts S., Majerus V. & Kinet J.M. (1999) NaCl effects on proline metabolism in rice (*Oryza sativa*) seedlings. *Physiologia Plantarum* **105**, 405–458.
- Maggio A., Bressan R.A., Hasegawa P.M. & Loco R.D. (1997) Moderately increased constitutive proline does not alter osmotic stress tolerance. *Physiologia Plantarum* **101**, 240–246.
- Mani S., Van de Cotte B., Van Montagu M. & Verbruggen N. (2002) Altered levels of proline dehydrogenase cause hypersensitivity to proline and its analogs in *Arabidopsis*. *Plant Physiology* **128**, 73–83.
- Munns R. (2002) Comparative physiology of salt and water stress. *Plant, Cell and Environment* **25**, 239–250.
- Murashige T. & Skoog F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum* **15**, 473–497.
- Nakashima K., Satoh R., Kiyosue T., Yamaguchi-Shinozaki K. & Shinozaki K. (1998) A gene encoding proline dehydrogenase is not only induced by proline and hypoosmolarity, but is also developmentally regulated in the reproductive organs of *Arabidopsis*. *Plant Physiology* **118**, 1233–1241.
- Nanjo T., Kobayashi M., Yoshida Y., Kakubari Y., Yamaguchi-Shinozaki K. & Shinozaki K. (1999) Antisense suppression of proline degradation improves tolerance to freezing and salinity in *Arabidopsis thaliana*. *FEBS Letters* **461**, 205–210.
- Nanjo T., Fujita M., Seki M., Kato M., Tabata S. & Shinozaki K. (2003) Toxicity of free proline revealed in an *Arabidopsis* T-DNA-tagged mutant deficient in proline dehydrogenase. *Plant Cell and Physiology* **44**, 541–548.
- Neff M.M. & Chory J. (1998) Genetic interactions between phytochrome A, phytochrome B, and cryptochrome 1 during *Arabidopsis* development. *Plant Physiology* **118**, 27–36.
- Ort D.R., Oxborough K. & Wise R.R. (1994) Depressions of photosynthesis in crops with water deficits. In *Photoinhibition of Photosynthesis from Molecular Mechanisms to the Field* (eds N.R. Baker & J.R. Bowyer), pp. 315–329. Bios Scientific Publishers, Oxford, UK.
- Peng Z., Lu Q. & Verma D.P.S. (1996) Reciprocal regulation of Δ^1 -pyrroline-5-carboxylate synthetase and proline dehydrogenase genes controls proline levels during and after osmotic stress in plants. *Molecular Genetics and Genomics* **253**, 334–341.
- Perez-Alfocea F., Balibrea M.E., Alarcon J.J. & Bolarin M.C. (2000) Composition of xylem and phloem exudates in relation to the salt tolerance of domestic and wild tomato species. *Journal of Plant Physiology* **156**, 367–374.
- Rajendrakumar C.S.V., Reddy B.V.D. & Reddy A.R. (1994) Proline–protein interactions: protection of structural and functional integrity of M_4 lactate dehydrogenase. *Biochemistry and Biophysics Research Communications* **201**, 957–963.
- Seki M., Narusaka M., Ishida J., *et al.* (2002) Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold and high salinity stresses using a full-length cDNA microarray. *Plant Cell* **31**, 279–292.
- Shi H., Ishitani M., Kim C. & Zhu J.-K. (2000) The *Arabidopsis thaliana* salt tolerance gene *SOS1* encodes a putative Na⁺/H⁺ antiporter. *Proceedings of the National Academy of Sciences of the USA* **97**, 6896–6901.
- Shi H., Quintero F.J., Pardo J.M. & Zhu J.-K. (2002) The putative plasma membrane Na⁺/H⁺ antiporter SOS1 controls long-distance Na⁺ transport in plants. *Plant Cell* **14**, 465–477.
- Shi H., Lee B.H., Wu S.J. & Zhu J.-K. (2003) Overexpression of a plasma membrane Na⁺/H⁺ antiporter gene improves salt tolerance in *Arabidopsis thaliana*. *Nature Biotechnology* **21**, 81–85.
- Shinozaki K., Shinozaki-Yamaguchi K. & Seki M. (2003) Regulatory network of gene expression in the drought and cold stress responses. *Current Opinion in Plant Biology* **6**, 410–417.
- Smirnov N. & Cumbes Q.J. (1989) Hydroxyl radical scavenging activity of compatible solutes. *Phytochemistry* **28**, 1057–1060.
- Strizhov N., Abraham E., Okresch L., Blickling S., Zilberstein A., Schell J., Koncz C. & Szabados L. (1997) Differential expression of two *P5CS* genes controlling proline accumulation during salt-stress requires ABA and is regulated by *ABA1*, *ABII* and *AXR2* in *Arabidopsis*. *Plant Journal* **12**, 557–569.
- Taji T., Seki M., Satou M., Sakurai T., Kobayashi M., Ishiyama K., Narusaka Y., Narusaka M., Zhu J.-K. & Shinozaki K. (2004) Comparative genomics in salt tolerance between *Arabidopsis* and *Arabidopsis*-related halophyte salt cress using *Arabidopsis* microarray. *Plant Physiology* **135**, 1697–1709.
- Tester M. & Davenport R. (2003) Na⁺ tolerance and Na⁺ transport in higher plants. *Annals of Botany* **91**, 1–25.
- Tsugane K., Kobayashi K., Niwa Y., Ohba Y., Wada K. & Kobayashi H. (1999) A recessive *Arabidopsis* mutant that grows photoautotrophically under salt stress shows enhanced active oxygen detoxification. *Plant Cell* **11**, 1195–1206.
- Vera-Estrella R., Barkla B.J., Garcia-Ramirez L. & Pantoja O. (2005) Salt stress in *Thellungiella halophila* activates Na⁺ transport mechanisms required for salinity tolerance. *Plant Physiology* **139**, 1507–1517.
- Verbruggen N., Hua X.-J., May M. & Van Montagu M. (1996) Environmental and developmental signals modulate proline homeostasis: evidence for a negative transcriptional regulator. *Proceedings of the National Academy of Sciences of the USA* **93**, 8787–8791.
- Volkov V., Wang B., Dominy P.J., Fricke W. & Amtmann A. (2004) *Thellungiella halophila*, a salt-tolerant relative of *Arabidopsis thaliana*, possesses effective mechanisms to discriminate between potassium and sodium. *Plant, Cell and Environment* **27**, 1–14.
- Wang Z.-L., Li P.-H., Fredricksen M., *et al.* (2004) Expressed sequence tags from *Thellungiella halophila*, a new model to study plant salt-tolerance. *Plant Science* **166**, 609–616.
- Ward J.M., Hirschi K.D. & Sze H. (2003) Plants pass the salt. *Trends in Plant Science* **8**, 200–201.
- Wessler S., Bureau T. & White S. (1995) LTR-retrotransposons and MITES: important players in the evolution of plant genomes. *Current Opinion in Genetics and Development* **5**, 814–821.
- Wong C.E., Li Y., Whitty B.R., D'Áz-Camino C., Akhter S.R., Brande J.E., Golding G.B., Weretilnyk E.A., Moffatt B.A. & Griffith M. (2005) Expressed sequence tags from the Yukon ecotype of *Thellungiella* reveal that gene expression in response to cold, drought and salinity shows little overlap. *Plant Molecular Biology* **58**, 561–574.
- Xiong L. & Zhu J.-K. (2002a) Salt tolerance. In *The Arabidopsis Book* (eds C.R. Somerville & E.M. Meyerowitz). American Society of Plant Biologists, Rockville, MD, USA. doi: 10.1199/tab.0048, URL <http://www.aspb.org/publications/arabidopsis/> [accessed on 5 April 2005]

Xiong L. & Zhu J.-K. (2002b) Molecular and genetic aspects of plant responses to osmotic stress. *Plant, Cell and Environment* **25**, 131–139.

Zhu J.-K. (2000) Genetic analysis of plant salt tolerance using *Arabidopsis*. *Plant Physiology* **124**, 941–948.

Zhu J.-K. (2001) Plant salt tolerance. *Trends in Plant Science* **6**, 66–72.

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