

RESEARCH PAPER

# A comparative study of salt tolerance parameters in 11 wild relatives of *Arabidopsis thaliana*

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## Abstract

Salinity is an abiotic stress that limits both yield and the expansion of agricultural crops to new areas. In the last 20 years our basic understanding of the mechanisms underlying plant tolerance and adaptation to saline environments has greatly improved owing to active development of advanced tools in molecular, genomics, and bioinformatics analyses. However, the full potential of investigative power has not been fully exploited, because the use of halophytes as model systems in plant salt tolerance research is largely neglected. The recent introduction of halophytic *Arabidopsis*-Relative Model Species (ARMS) has begun to compare and relate several unique genetic resources to the well-developed *Arabidopsis* model. In a search for candidates to begin to understand, through genetic analyses, the biological bases of salt tolerance, 11 wild relatives of *Arabidopsis thaliana* were compared: *Barbarea verna*, *Capsella bursa-pastoris*, *Hirschfeldia incana*, *Lepidium densiflorum*, *Malcolmia triloba*, *Lepidium virginicum*, *Descurainia pinnata*, *Sisymbrium officinale*, *Thellungiella parvula*, *Thellungiella salsuginea* (previously *T. halophila*), and *Thlaspi arvense*. Among these species, highly salt-tolerant (*L. densiflorum* and *L. virginicum*) and moderately salt-tolerant (*M. triloba* and *H. incana*) species were identified. Only *T. parvula* revealed a true halophytic habitus, comparable to the better studied *Thellungiella salsuginea*. Major differences in growth, water transport properties, and ion accumulation are observed and discussed to describe the distinctive traits and physiological responses that can now be studied genetically in salt stress research.

**Key words:** Germination, halophytes, ion contents, root elongation, stomata, water relations.

## Introduction

Elucidation of the fundamental mechanisms underlying plant salt tolerance has historically been based on comparative analyses between halophytic and glycophytic species. The ultimate objective of these analyses has been to understand how the former deal with salt and to identify critical salt tolerance traits that could potentially be used in agricultural crops that are almost exclusively glycophytes. However, the seemingly obvious positive outcome of this approach has been greatly limited by the lack of information on the genetic bases for salt tolerance in halophytes. In fact, genetic studies using halophytic species are virtually non-existent (Munns and Tester, 2008), and the potential of this resource of natural salt tolerance remains essentially unexplored (Cushman *et al.*, 1989; Flowers and Yeo, 1995; Kant *et al.*, 2006; Flowers and Colmer, 2008; Amtmann, 2009). In order to exploit genetically the existing resources, it is necessary to identify species that are halophytic and are either amenable to genetic analysis or exhibit characteristics of an established genetic model system. With a few exceptions (Dassanayake *et al.*, 2009), after decades of study using halophyte models such as *Mesembryanthemum*, *Salicornia*, *Spergularia*, *Limonium*, *Distichlis*, or various mangroves, no genetic approach has resulted that advanced these models. Over the last two decades the use of *Arabidopsis thaliana* as a genetic model system has advanced plant biology to new levels of understanding (Meinke *et al.*, 1998; Sanders, 2000; Chen *et al.*, 2004). Although *Arabidopsis*, a salt-sensitive species, can provide only limited information about mechanisms that support salinity tolerance, numerous genes involved in salt tolerance have been revealed by mutational approaches that resulted in plants with an even lower salt tolerance (Sanders, 2000). Much has been learned from this approach, yet these studies fail to reveal the genetic bases of extreme salt tolerance exhibited by natural halophytes. In order to understand the genetic bases that characterizes halophytism better, it is necessary to establish 'halophyte genetic model systems' (as advocated by Flowers and Colmer, 2008) that can be manipulated with ease and flexibility comparable to that available for *Arabidopsis*. Such a genetic model, an Ar**a**bidopsis-Relative Model System (**ARMS**), could contribute to the identification and characterization of halophyte-specific mechanisms. A species in this category is *Thellungiella salsa* (salt cress, previously termed *T. halophila*) (Bressan *et al.*, 2001; Inan *et al.*, 2004; Amtmann *et al.*, 2005; Amtmann, 2009). *Thellungiella parvula* has now been added. Both are close relatives of *Arabidopsis*, and genetic and genomic resources exist and/or are being generated at present ([www.thellungiella.org](http://www.thellungiella.org)). Comparative studies between salinity stress adaptation in *Arabidopsis* and its relatives have provided insights into the genetic bases of halophytism (Inan *et al.*, 2004; Taji *et al.*, 2004; Wang *et al.*, 2004, 2006; Gong *et al.*, 2005; Wong *et al.*, 2006; Oh *et al.*, 2009; Amtmann, 2009). Within the *Brassicaceae* family other species have been tested for their performance under abiotic stresses, including species of

*Hirschfeldia*, *Capsella*, *Thlaspi*, and *Lepidium* (Aksoy *et al.*, 1999; Pedras *et al.*, 2003; Davies *et al.*, 2004; Madejon *et al.*, 2005; Fischerova *et al.*, 2006; Fuentes *et al.*, 2006; Gisbert *et al.*, 2006; Jiménez-Ambríz *et al.*, 2007).

In this study side-by-side comparisons of responses to abiotic stresses by several species related to *Arabidopsis* are reported. Growth parameters, water, and ion homeostasis were primarily considered to link morphological and physiological modifications to individual stress adaptation mechanisms. The physiological/phenotypic characterization of abiotic stress responses are considered to be an essential prerequisite for understanding genetic and genomics-type studies that are being extended to some of these species at present.

## Materials and methods

### *Plant material and growth conditions*

Eleven wild relatives of *Arabidopsis thaliana*, belonging to the *Brassicaceae* were collected from different environments (e.g. seaside, desert land, waste sites, road embankments, and salt flats) and were identified with the help of Dr Al-Shehbaz, Missouri Botanical Garden (Table 1). After a preliminary assessment of their response to NaCl treatment, four species were chosen for further investigations: *Thellungiella salsa* (ecotype Shandong), *Thellungiella parvula*, *Lepidium virginicum*, and *Descurainia pinnata*. *Arabidopsis thaliana* (ecotype Col-0) was used as the glycophytic reference species in all experiments.

Unless otherwise specified, for *in vivo* experiments plants were sown in plastic flats containing Metro Mix 360 pot medium (Scotts-Sierra, Marysville, OH) and grown in a greenhouse under 21/8 °C day/night temperatures with a 16 h photoperiod. One week prior to NaCl treatments, seedlings were transferred into 7.5 cm pots filled with artificial soil, Turface<sup>®</sup> calcined (Profile Products, Buffalo Grove, IL). Plants were placed in a growth chamber with a photosynthetic photon flux of 250 μmol m<sup>-2</sup> s<sup>-1</sup> from cool-white fluorescent bulbs and a 16 h photoperiod. Day and night temperatures were set at 22 °C and 19 °C, respectively. Plants were irrigated with nutrient solution containing 200 mg N l<sup>-1</sup> supplied from a 1000 mg l<sup>-1</sup> 15-5-15 commercial fertilizer formulation (Miracle Gro<sup>®</sup> Excel<sup>®</sup> Cal-Mag; The Scotts Co., Marysville, OH) every other day. NaCl was added to the nutrient solution at the desired concentration or by incremental increases until the final desired concentrations were reached. The hydroponic system was deliberately not used, since not all species respond well to this system and in our case (a comparison of 11 species) could have introduced a further source of variability. In addition, continuous measurements of transpiration fluxes cannot be done with hydroponics since the necessary aeration of the nutrient solution would affect the measurements of the plants on the scale (over a 5 d period).

Seeds used for germination and root bending experiments were briefly surface-sterilized in a solution of 70% (v/v) ethanol, followed by 30% (v/v) commercial bleach solution for 10 min. They were then washed with sterilized water four times and suspended in sterile 0.1% (w/v) low-melting agarose before plating on Murashige and Skoog (MS) agar Petri dishes. Plates were stored at 4 °C for 48 h to synchronize germination and then incubated in a growth chamber with 16 h of light at 22 °C and 8 h of darkness at 18 °C.

### *NaCl treatments and growth measurements*

Starting 25 d after sowing (DAS), plants were watered with 150 mM NaCl for 30 d. At the end of the experiment, plants were

**Table 1.** Brassicaceae species considered in this study.

Species	Common name	Ploidy	Specimen collection location	Species habitat	Genus /Species native distribution
<i>Arabidopsis thaliana</i>	Mouse-ear-cress, rockcress	n=5 2n=10	Originally from Europe	Wooded hillsides, waste places	Europe and Asia
<i>Barbarea verna</i>	Yellow flower, winter cress	n=8 2n=16	Coastal regions of Fujien South China	Damp soil, roadsides and waste places	Eastern Europe and southwestern Asia
<i>Capsella bursa-pastoris</i>	Shepherd's purse	n=8, 16 2n=16, 32	Sea coast of South Korea	Waste areas, road margins	All continents, except Antarctica
<i>Descurainia pinnata</i> subsp. <i>glabra</i>	Western tansy mustard	n=7 2n=28	Desert of North Africa, Marocco	Sandy fields, gravel, white saline areas, dunes, open desert	Desert regions from Nevada to central and northwestern Mexico, Africa
<i>Hirshfeldia incana</i>	Conil yellow, Mediterranean hoary-mustard	n=7 2n=14	Sea coast of South-West Spain	Waste places, roadsides and canyons	Mediterranean region
<i>Lepidium densiflorum</i>	Common pepperweed, prairie peppergrass	n=16 2n=32	Byron Bay, sea coast of eastern Australia	Sandy soil, waste places	All continents, except Antarctica
<i>Lepidium virginicum</i>	Virginia pepperweed		Byron Bay, sea coast of eastern Australia	Coastal regions, sea cliffs, dry creek beds, dry plains	All continents, except Antarctica
<i>Malcolmia triloba</i>	Conil blue	n=7, 14 2n=28	Sea coast of South-West Spain	Waste and disturbed areas, gravel pits	Asia and Mediterranean region
<i>Sisymbrium officinale</i>	Hedge mustard	n=7 2n=14	Sea coast, Sorrento, Italy	Disturbed sites	Europe
<i>Thellungiella halophila</i> * ( <i>Thellungiella salsuginea</i> )	Salt cress	n=7 2n=14	Shandong province, sea coast of North eastern China	Sea coasts, sandy alkaline soils	Asia, Central Canada to Colorado
<i>Thellungiella parvula</i>		n=7 2n=14	Central Turkey, dry lake beds	Salt flats of ancient lakes and river beds	Central Asia, Southern Russia, Turkey
<i>Thlaspi arvense</i>	Penny-cress	n=7 2n=14	Lafayette, IN, USA	Roadsides, waste places	Central Europe to western Asia

\* For systematics analyses and the position of *T. salsuginea*, see Rollins 1993; Al-Shehbaz and O'Kane 1995.

collected for measurements of root length and leaf area, using Image J® software (Abramoff *et al.*, 2004). Five plants per treatment (0 and 150 mM NaCl) were considered, with three replicates. Data were normalized against control (0 mM NaCl).

For the determination of the LD50<sub>NaCl</sub> (NaCl concentration in the nutrient medium that is lethal to 50% of the population), 12 salt treatments were imposed (0, 50, 100, 150, 200, 250, 300, 350, 400, 450 500, and 600 mM NaCl) by the incremental increase of 50 mM NaCl every 2 d, starting from 30 DAS. The experiment lasted 30 d and those plants that survived were counted at the end of the experiment, from pools of 20 plants/species/treatment, with three replicates. Plants that showed irreversible wilting, generally followed by necrosis on all leaves were considered to be dead.

#### Germination assay

Seeds were surface-sterilized and sown on Petri dishes containing either MS agar medium or MS medium supplemented with 150 mM NaCl. Seeds were stratified at 4 °C for 4 d and transferred to a growth chamber with 16 h of light at 22 °C and 8 h of darkness at 18 °C. The number of germinated seeds was assessed 14 d after sowing on plates containing 10 seeds per species, with three replicates.

#### Root elongation measurements

Seeds were surface-sterilized and plated on MS agar covered with a cellophane membrane (Bio-Rad). Ten seeds per genotype were sown in each plate and 12 plate repetitions were considered. Petri plates were then placed vertically in the growth chamber according to Verslues *et al.* (2006). After 1 week, seedlings were transferred to new Petri dishes containing 0 or 300 mM NaCl. Plates were kept vertically and rotated 180° to visualize new root growth

(Root Bending Assay; Verslues *et al.*, 2006). After 10 d, photographs of the dishes were collected using a transmission scanner. Roots were then measured using Image J software (Abramoff *et al.*, 2004).

#### Leaf water relations

Forty days after sowing, four single-plant pots per genotype were sealed with a plastic film to prevent water loss from the soil surface, leaving the shoot protruding from the film. Before sealing, plants were watered to capacity with water (control) or water plus 300 mM NaCl (in plants acclimated with water plus 50 mM NaCl for 2 d and water plus 100 mM NaCl for an additional 2 d). Each pot was then placed on an electronic balance under a light intensity of 140 μmol m<sup>-2</sup> s<sup>-1</sup> at 25 °C. After approximately 35 h of further acclimation in the growth chamber, weight loss was automatically measured every hour for 5 d using PC software. Water loss values were normalized for plant dry weights taken at the end of the experiment.

#### Stomatal size and density

Stomatal size and density were measured using a bright-field light microscope. Leaf surface imprints of non-salinized control plants were obtained by using transparent nail polish. Imprints were taken from the middle portion of the blade between the midrib and the leaf margin, on three leaves of comparable age per species, with 20 measurements per leaf.

#### Na<sup>+</sup> and K<sup>+</sup> ion contents

Three-week-old *A. thaliana* (ecotype Col-0), *T. salsuginea* (ecotype Shandong), and *T. parvula* plants were grown as described above.

The NaCl treatments were applied by incremental increases of NaCl in the irrigation water, every 7 d, until final concentrations of 0, 100, 200, 300, and 500 mM NaCl were reached. For *T. salsuginea* and *T. parvula*, concentrations were incremented at 100 mM intervals, while 50 mM increments were used for *A. thaliana*. Plants were harvested 28 d and 42 d after reaching the final salt treatment.

At harvest, seedlings were rinsed with deionized water and dried at 65 °C for 2 d. One hundred milligrams of dry leaf material was then extracted with 10 ml of 0.1 M HNO<sub>3</sub> for 30 min and then filtered through Whatman no.1 filter paper. Na<sup>+</sup> and K<sup>+</sup> contents in the solutions were determined with a Varian Spectra AA-10 atomic absorption spectrophotometer (Varian Techtron Pty. Ltd., Mulgrave, Victoria, Australia).

Data were analysed by ANOVA and means were compared with the least significance difference (LSD) test where indicated.

## Results

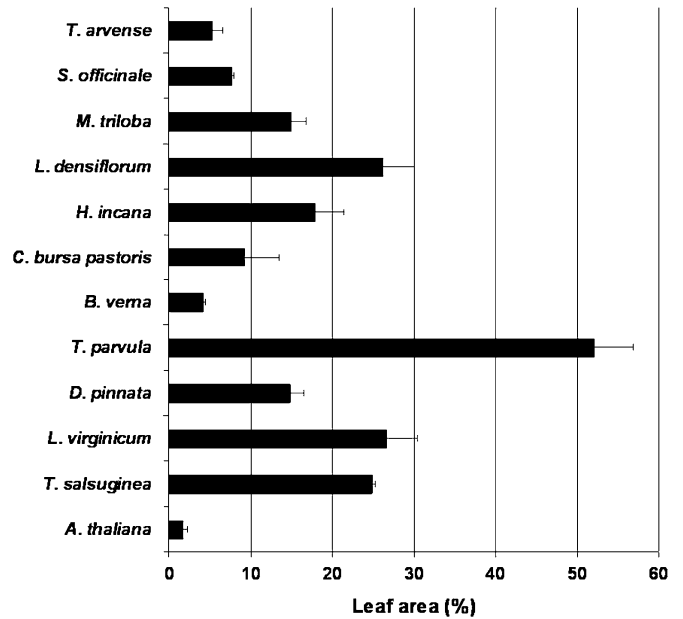
### Morphology and life cycle

The species selected share many important features with *Arabidopsis*. The 11 species belong to the *Brassicaceae* and their life cycles can be completed in 6–12 weeks. Some of the species (*T. salsuginea* and *D. pinnata*) showed slower growth compared with *Arabidopsis*, while others (*L. virginicum* and *T. parvula*) displayed higher growth rates and reached a much larger size relative to *Arabidopsis*. No differences in leaf pubescence or other xerophytic traits were observed with the exception of a slightly more pronounced leaf succulence of *D. pinnata* (data not shown).

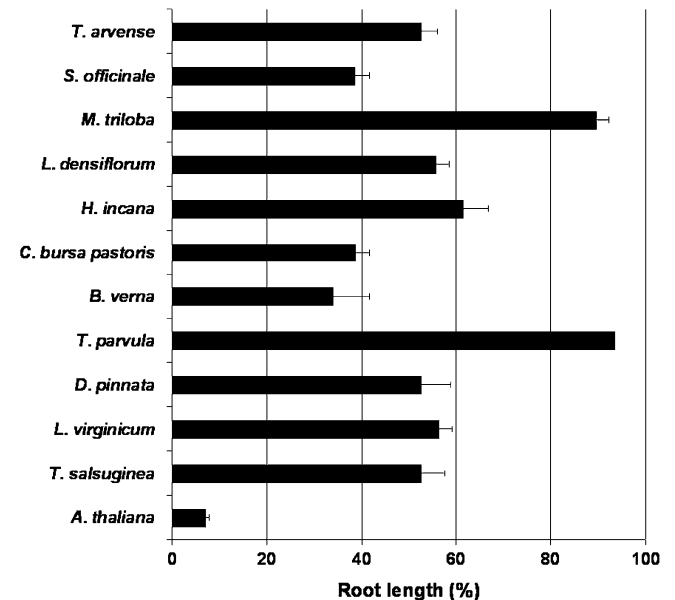
### Growth response to salt stress

A first comparison between different species was aimed at assessing their performance in saline environments in terms of both general growth and survival. *A. thaliana* and *T. salsuginea*, the latter known to tolerate very high NaCl concentrations (Inan et al., 2004), were used as controls. Under the imposed experimental conditions at 150 mM NaCl leaf area was significantly reduced by the stress in *A. thaliana*, whereas *L. densiflorum* and *L. virginicum* were comparable to *T. salsuginea*. By contrast, higher relative leaf areas were observed for *T. parvula* (Fig. 1). Similarly, *Arabidopsis* root growth was significantly inhibited at 150 mM NaCl. Root growth of *T. arvense*, *L. densiflorum*, *H. incana*, *D. pinnata*, and *L. virginicum* was less affected by salinity compared to *Arabidopsis* with a response comparable to *T. salsuginea*. Significantly tolerant root systems were found for *M. triloba* and *T. parvula*. Both were practically unaffected by this NaCl concentration (Fig. 2).

The NaCl lethal dose to 50% of the population (LD<sub>50</sub><sub>NaCl</sub>) was used to assess plant survival to salt stress. Most species revealed their halophytic nature since they had a survival threshold in the range between 200 and 400 mM NaCl. This was much higher than *Arabidopsis*, whose LD<sub>50</sub><sub>NaCl</sub> was 150 mM. An LD<sub>50</sub><sub>NaCl</sub> of 500 mM NaCl was measured for *L. virginicum* and *L. densiflorum*, whereas the highest tolerance (600 mM) was found for *T. salsuginea*, *M. triloba*, and *T. parvula* (Fig. 3). Dose–response curves, however, did not always reveal a typical sigmoidal shape,

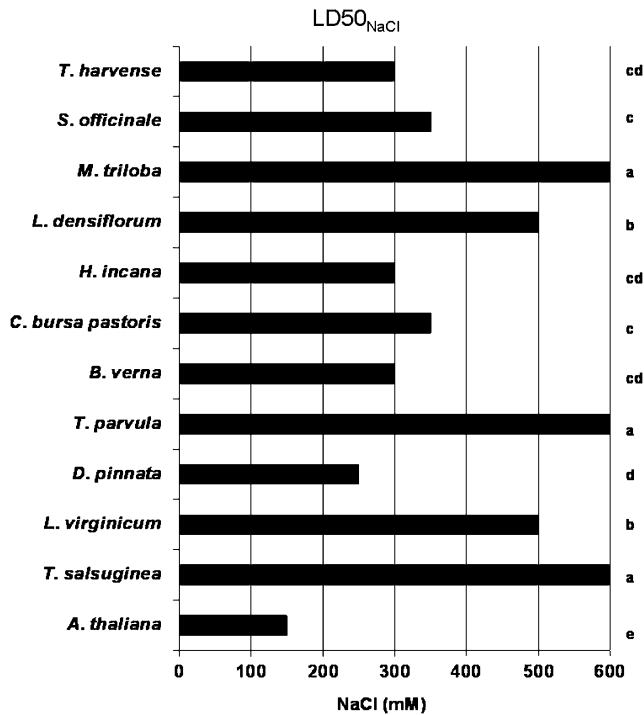


**Fig. 1.** Effect of 150 mM NaCl stress on leaf area of different *Brassicaceae* species. Salt treatment started 25 d after sowing and lasted for 30 d. Leaf area was measured using a scanner and image processing software over a sample of five plants per species, with three replicates. Values are expressed as a percentage (%) of leaf area in non-salinized plants. Values are means  $\pm$  SE ( $n=15$ ).



**Fig. 2.** Effect of 150 mM NaCl on primary root length of different *Brassicaceae* species. Salt treatment started 25 d after sowing and lasted 30 d. Root length was measured using a scanner and image processing software over a sample of five plants per species, with three replicates. Values are expressed as a percentage (%) of root length in non-salinized plants. Values are means  $\pm$  SE ( $n=15$ ).

which may have introduced some errors in our assessment. In some cases (e.g. *T. arvense* and *B. verna*), a two-step behaviour was observed, suggesting the existence of two tolerance mechanisms, one allowing approximately 100%



**Fig. 3.** Species-specific lethal dose for 50% of the population (LD<sub>50</sub>) under NaCl stress in different *Brassicaceae* species. Salt treatments ranged from 0–600 mM NaCl, started 30 d after sowing and lasted for 30 d. Survival rates were assessed at the end of the treatments over samples of 20 plants, with three replicates. Letters indicate differences at  $P < 0.05$  ( $n=60$ ).

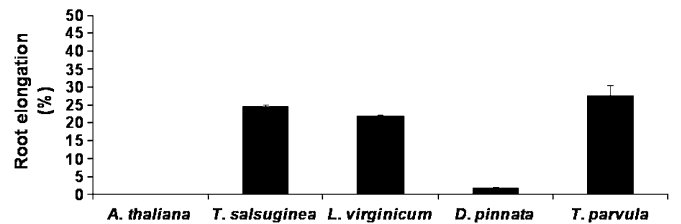
survival at low salt concentrations and the other one allowing 50–60% survival at higher salt concentrations (see Supplementary Fig. S1 at *JXB* online).

Based on these initial measurements, *L. virginicum*, *D. pinnata*, and *T. parvula* were selected for further analyses. *M. triloba* indeed ranked high in terms of LD<sub>50<sub>NaCl</sub></sub>, however it had a very high root-to-shoot ratio in response to salinity with a dramatic reduction in shoot development, clearly representing a fundamentally different stress response than the other species. For this reason it was not included in subsequent experiments.

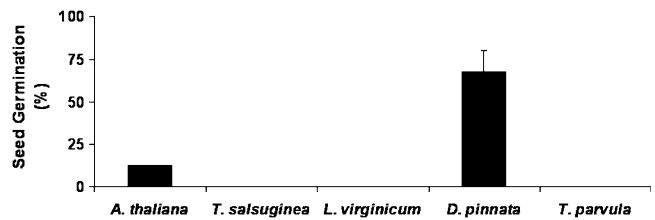
#### Root bending assay and germination

The results of this and subsequent sections refer to the three selected novel ARMS, *L. virginicum*, *D. pinnata*, and *T. parvula*, and the two controls, *A. thaliana* and *T. salsuginea*. To confirm the growth performance under saline conditions, root growth was assessed by the root bending assay (Verslues *et al.*, 2006). At 300 mM NaCl the growth of *A. thaliana* had stopped, whereas slight further growth was observed in *D. pinnata*. Growth rates comparable to *T. salsuginea* were observed in *T. parvula* and *L. virginicum* (Fig. 4).

Consistent with results reported by Inan *et al.* (2004), the best performers with respect to growth under salt had a low germination rate in a saline environment, behaviour that is shared by many halophytes (Fig. 5). *T. salsuginea*,



**Fig. 4.** Effect of 300 mM NaCl stress on root elongation of selected *Brassicaceae* species. Five days after germination on regular MS medium, with plates positioned vertically, seedlings were moved to salt. The plates were then rotated 180° to visualize further growth (root bending assay). Measurements were assessed after 10 d on 10 plants per species, with three replicates. Values are expressed as a percentage (%) of growth measured in non-salinized plants. Values are means  $\pm$  SE ( $n=30$ ).



**Fig. 5.** Effect of 150 mM NaCl on seed germination of selected *Brassicaceae* species. Germination rate was determined 14 d after sowing on plates containing 10 seeds per species, with three replicates. Values are expressed as a percentage (%) of germination as compared with non-salinized plates. Values are means  $\pm$  SE ( $n=30$ ).

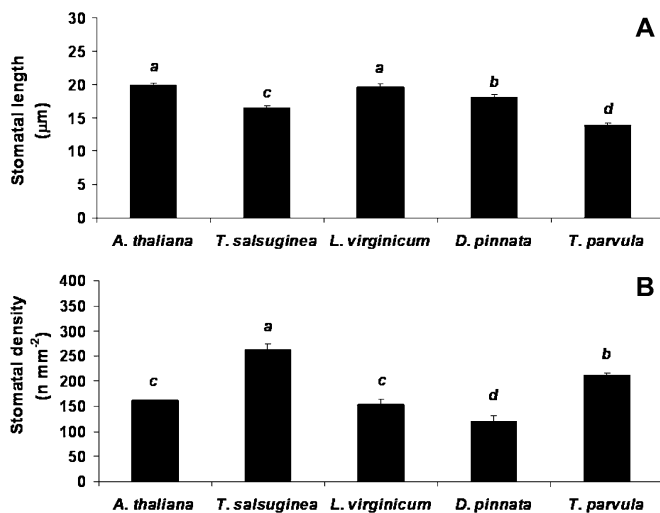
*L. virginicum*, and *T. parvula* were unable to germinate at 150 mM NaCl, while the germination rate of *D. pinnata* (67.4%) was higher than that of *A. thaliana* (12.6%). The germination rate in the absence of salt was very similar in *A. thaliana* and *T. parvula*, whereas it was about 80% lower in *T. salsuginea* (data not shown). Germination hypersensitivity to NaCl has been reported for salt cress (Inan *et al.*, 2004) and seeds of several other halophytes (Flowers *et al.*, 1986). The delayed germination reported for some halophytes has been viewed as an associated protective strategy to ensure maximal survival (Inan *et al.*, 2004). During the experiments, it was not assessed whether the absence of germination after 14 d was due to this phenomenon or to irreversible damage by NaCl at the early developmental stages. However, hypersensitivity of salt cress seed germination to ABA suggests that increased dormancy mediates the low germination rate (Inan *et al.*, 2004).

#### Stomatal characteristics and plant water use

Differences in stomatal size and density were found among the five species under assessment (Fig. 6A, B). The stomata of these plants were very similar in width (shorter axis), whereas major differences were found in terms of stomatal length (longer axis). The shortest stomata (Fig. 6A) were detected in *T. parvula* and *T. salsuginea*, whereas the stomata of *L. virginicum* and *A. thaliana* were significantly

longer than those in *D. pinnata*. Interestingly, lower stomatal size was correlated to higher stomatal density (Fig. 6B).

Measurement of daily fluctuations of transpiration over 5 d confirmed (Lovelock and Ball, 2002) that the transpiration rate of halophytic species was generally lower ( $\approx 60\%$ ) than that of the glycophytic control (*A. thaliana*) in the absence of NaCl (Fig. 7). The upper limits of stomatal aperture and the amplitude (max–min) of the daily transpiration flux were lower in *T. salsuginea*, *L. virginicum*, *D. pinnata*, and *T. parvula* in comparison to *A. thaliana*. In the response to salinity the amplitude of the daily fluctuations were reduced in all plants, but this reduction was relatively less pronounced in the halophytic species compared with *A. thaliana*. *L. virginicum* showed almost no reduction of the transpiration water flux, whereas the day–night fluctuation was nearly abolished in *D. pinnata*. The relative water loss by plants stressed at 300 mM NaCl, as compared with non-salinized plants, was highest in *L. virginicum* and lowest in *T. salsuginea*, *D. pinnata*, and *A. thaliana* (Fig. 8). Overall, some species had a particularly low transpiration rate, below  $1 \text{ g H}_2\text{O loss g}^{-1} \text{ DW h}^{-1}$ , namely *D. pinnata*, *L. virginicum*, and *T. salsuginea*. Transpiration rates were just over  $1 \text{ g H}_2\text{O loss g}^{-1} \text{ DW h}^{-1}$  in *T. parvula*. The latter species also exhibited best performance under salt stress in terms of leaf area, root development and  $\text{LD}_{50\text{NaCl}}$ . Finally, high transpiration rates, over  $3 \text{ g H}_2\text{O loss g}^{-1} \text{ DW h}^{-1}$  were recorded for *A. thaliana*. Overall, halophytes transpire less in the absence of stress and are, in general, relatively less affected by salt stress in terms of transpiration, compared to glycophytes (Figs 7, 8). These differences indicated that a low transpiration in halophytes in comparison to glycophytes is one of the outstanding physiological mechanisms that may lead to stress tolerance in extremophile species, in which a balanced control of growth signals, detoxification mechanisms, and ion/water

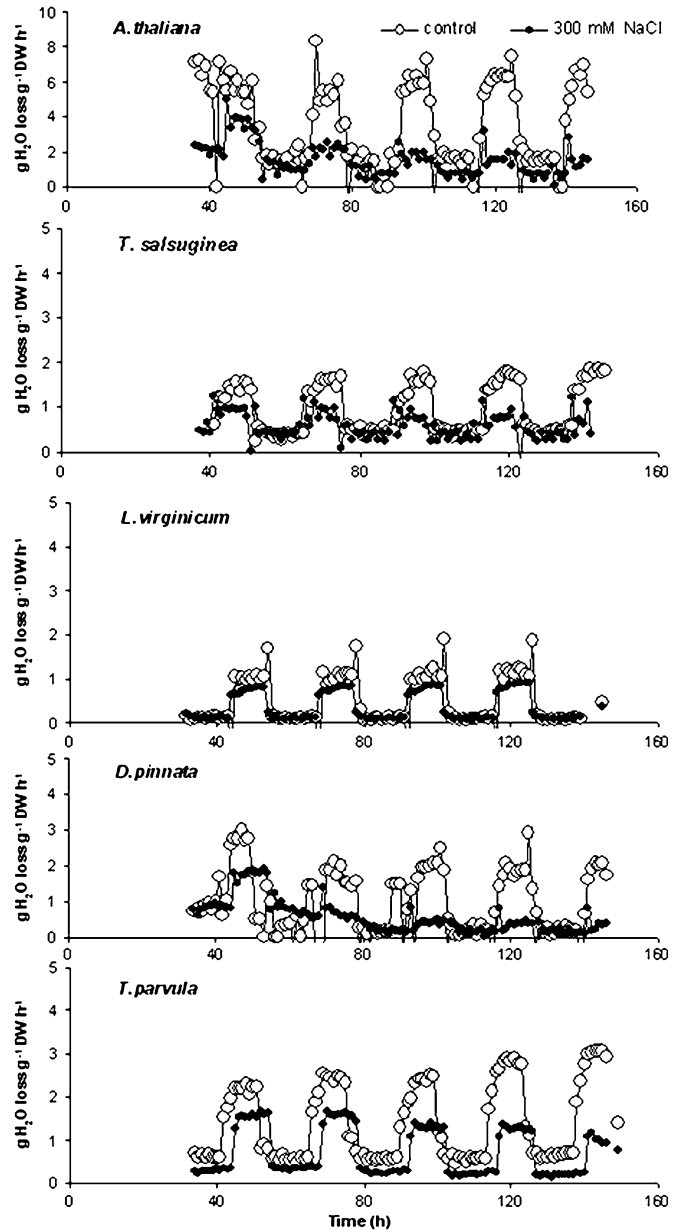


**Fig. 6.** Stomatal length (A) and density (B) of selected *Brassicaceae* species. Values refer to 20 independent measures per leaf on three leaves per species (non-salinized control plants). Letters indicate differences at  $P < 0.05$  ( $n=60$ ).

homeostasis must be orchestrated through the genetic structure of these species.

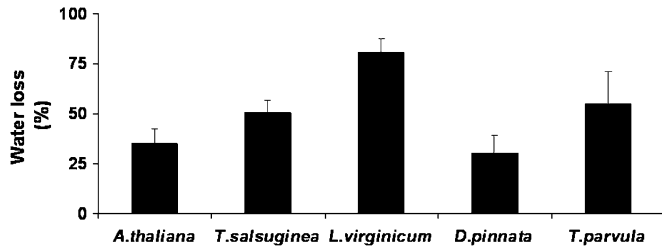
### Ion contents

The pattern of  $\text{Na}^+$  and  $\text{K}^+$  accumulation in *Arabidopsis* and two of the halophytes under assessment is shown in Fig. 9. The accumulation of  $\text{Na}^+$  in *T. salsuginea* and *T. parvula* at increasing salinity was much lower than that



**Fig. 7.** Effect of salt stress on water loss in selected *Brassicaceae* species. Four-week-old seedlings grown under long-day conditions with cool-white fluorescent light were used for measurements of whole-plant water loss. Plants were grown singularly in 9 cm pots, which were sealed in plastic wrap and placed on electronic balances. Weight was determined every 60 min for 5 d. The experiment was repeated three times. Values are means of transpiration rates of four plants in the three independent experiments ( $n=12$ ). White circle, control; black diamonds, 300 mM NaCl.

observed in *Arabidopsis* at external concentrations between 0 and 200 mM NaCl. At higher salinity (300–500 mM NaCl) and longer exposure (42 d) *T. salsuginea* and *T. parvula* accumulated similar levels of Na<sup>+</sup>. *T. parvula* plants grown under control conditions contained exceptionally high K<sup>+</sup> such that, even after salinization, its concentration remained higher than that in the other species.



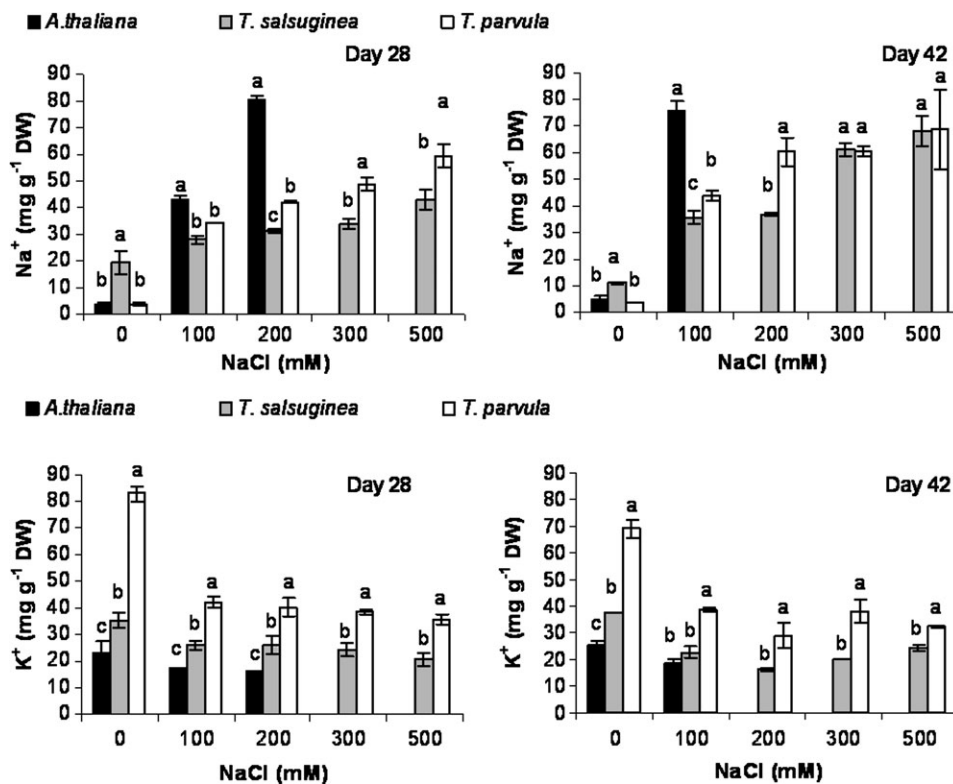
**Fig. 8.** Relative water loss in 300 mM NaCl stressed plants, as compared with non-salinized plants. Four-week-old seedlings grown under long-day conditions with cool-white fluorescent light were used for measurements of whole-plant water loss. Plants were grown singularly in 9 cm pots, which were sealed in plastic wrap and placed on electronic balances. Weight was determined every 60 min for 5 d. Values are a percentage (%) of transpiration rate as compared with non-salinized plants averaged over the 5 d. Values are means  $\pm$  SE ( $n=12$ ).

The concentration of K<sup>+</sup> remained virtually unaffected in both *Arabidopsis* and *T. salsuginea* at increasing salinity (0–500 mM), whereas a dramatic drop of the K<sup>+</sup> concentration was detected when plants of *T. parvula* were exposed to 100 mM NaCl. Consistently, different responses to increasing salinity were observed in *T. salsuginea* compared with *T. parvula* in terms of growth (Fig. 10). *T. parvula* was slightly more tolerant than *T. salsuginea* at moderate salinity (100 mM NaCl), yet at advanced salinization (200 and 300 mM) *T. salsuginea* was relatively more tolerant compared with *T. parvula*.

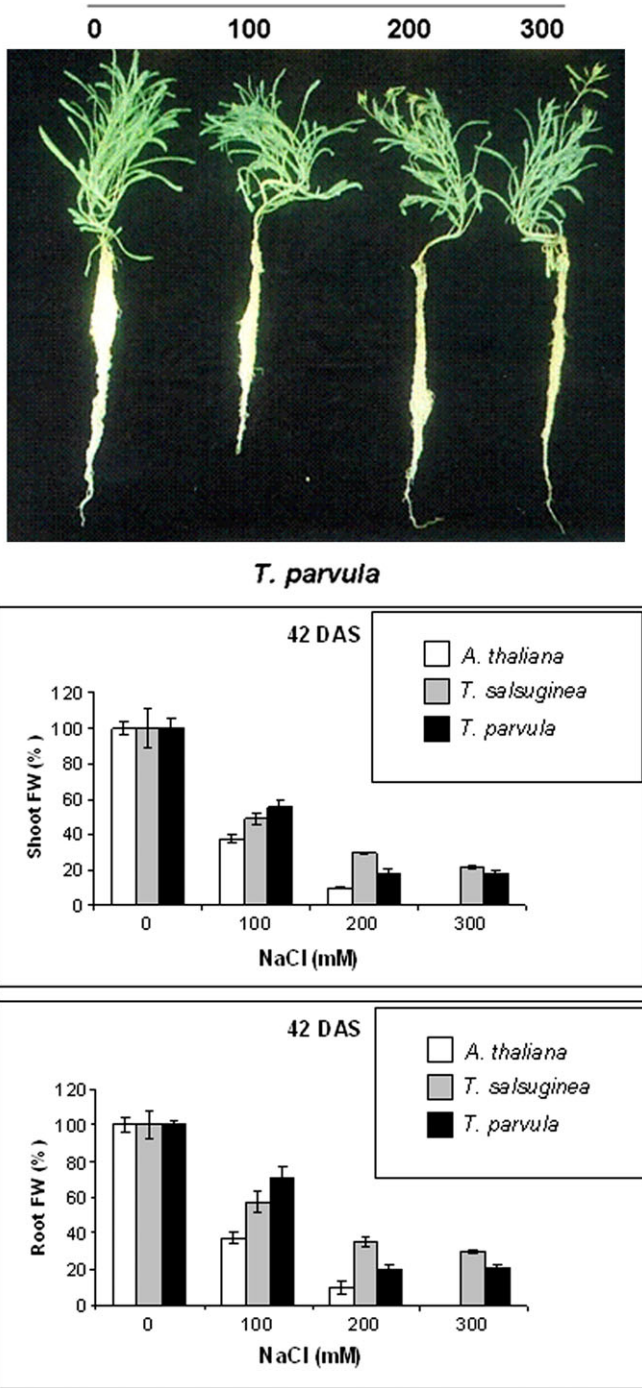
## Discussion

### Identification of novel ARMS species

The halophytic nature of 11 *Brassicaceae* species with growth habits similar to *Arabidopsis* was investigated to identify candidates suitable for further comparative genomic analysis. All species studied here displayed a significantly higher tolerance at 150 mM NaCl than *Arabidopsis*. However, significant variability in terms of leaf area and root development was found between species, ranging from 2 $\times$  to 25 $\times$  and from 4 $\times$  to 11 $\times$  the size of *Arabidopsis*, for leaf area and root length, respectively (Figs 1, 2). The



**Fig. 9.** Sodium and potassium concentrations in leaves of *Arabidopsis thaliana*, *Thellungiella halophila*, and *Thellungiella parvula*. NaCl treatments were applied by incremental increase of NaCl in the irrigation water until the final concentrations of 0, 100, 200, 300, and 500 mM NaCl were reached. Plants were harvested 28 d and 42 d after imposition of the final increase of the NaCl concentrations. At harvest, seedlings of treated and control plants were rinsed with deionized water and dried at 65 °C for 2 d and Na<sup>+</sup> and K<sup>+</sup> contents in the solutions were determined by using an atomic absorption spectrophotometer. Letters indicate differences at  $P < 0.05$  ( $n=2$ , for each of two samples four plants were ground).



**Fig. 10.** Effect on growth of different concentrations of NaCl in *Thellungiella parvula*. NaCl was increased incrementally in the irrigation water to final concentrations of 100, 150, and 200 mM for *A. thaliana* and 100, 200, and 300, for *T. halophila* and *T. parvula*. Shoots and roots were harvested at day 42 of stress. FW, fresh weight. Values are means  $\pm$  SE ( $n=5$ ).

species-specific lethal dose for NaCl that killed 50% of the population ( $LD_{50_{NaCl}}$ ) clustered these species into two major groups, one in the range between 200–400 mM NaCl, including *T. arvensis*, *H. incana*, *C. bursa pastoris*, *B. verna*, and *D. pinnata* and a second group with a  $LD_{50_{NaCl}}$  between 400–600 mM NaCl including *M. triloba*,

*L. densiflorum*, *T. parvula*, *L. virginicum*, and *T. salsuginea*. The survival at high NaCl concentrations (>400 mM NaCl for species in the second group) was consistent with that observed in many true halophytes. Based on the overall growth performance of these plants four categories of tolerance were identified: (i) halophytic habit (*T. salsuginea* and *T. parvula*), (ii) highly tolerant (*L. densiflorum* and *L. virginicum*), (iii) moderate tolerance (*M. triloba*, *H. incana*, *D. pinnata*), and (iv) marginally better than *Arabidopsis* (*T. arvensis*, *S. officinale*, *B. verna*). *D. pinnata*, *T. parvula*, and *L. virginicum* were selected for further analysis in comparison to *Arabidopsis* (glycophyte) and *T. salsuginea* (halophyte).

#### Morphological and physiological tolerance traits associated with transpiration and water transport

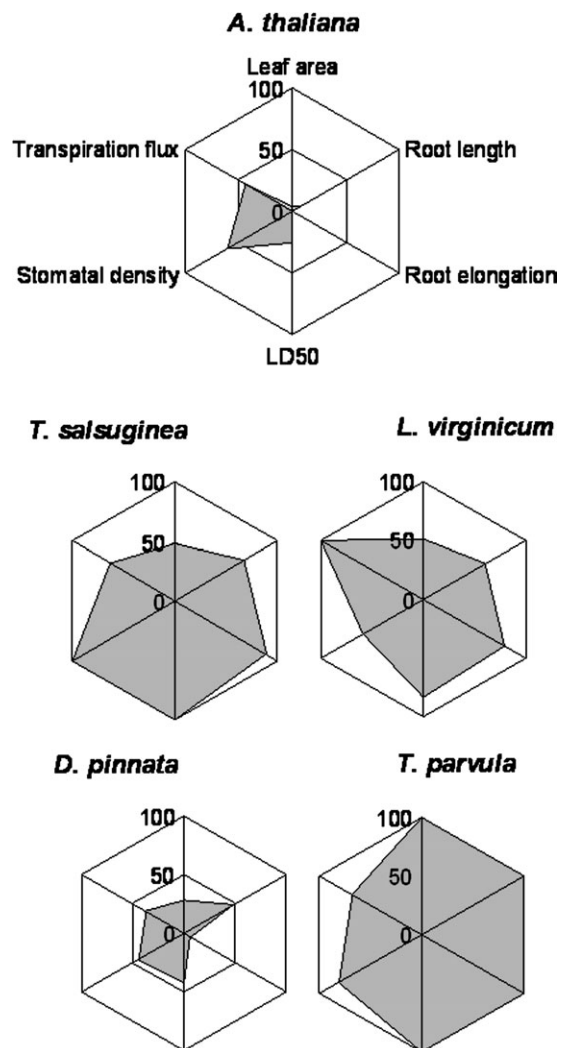
Leaf stomatal densities were higher in *T. salsuginea*, *L. virginicum*, *D. pinnata*, and *T. parvula* compared with *Arabidopsis*. Nevertheless, under both saline and non-saline conditions, the halophytic species exhibited a whole-plant day/night transpiration rate much lower than that observed for *Arabidopsis* (Fig. 7). This observation was in line with several reports that documented decreased stomatal conductance following salt exposure in halophytes (Lovelock and Ball, 2002; Boughalleb et al., 2009). Although the ability to control transpiration water flux versus growth (i.e. water use efficiency) is a critical tolerance determinant in both glycophytes and halophytes, a large body of literature on water relations in glycophytes exposed to stressful environments is mirrored by a rather limited number of studies available for halophytes (Glenn et al., 1999; Flowers and Colmer, 2008). Transport of salt to the shoot can be drastically influenced by stomatal function (Dalton et al., 2000; Lovelock and Ball, 2002) as confirmed by the large increase in the transitory tolerance of glycophytes that is observed when transpiration is inhibited. Several morphological and physiological mechanisms, such as the control of transpirational water flux (i.e. via stomatal and/or aquaporins regulation), that are associated with ion loading and accumulation, have been described and linked to specific genetic determinants (Di Lorenzo et al., 1996; Gray et al., 2000; Wang et al., 2001; Zhu et al., 2002). The stomatal density of *T. salsuginea* and *T. parvula* was highest among the species examined (Fig. 6). In these two species, the higher number correlated with a lower length of the individual stomata. This result confirms earlier studies by Inan et al. (2004), who reported a similar morphological character in *T. salsuginea* compared with *A. thaliana*, and the same was documented in other halophytes (Osmond et al., 1980; Perera et al., 1994). The transpiration flux of *T. salsuginea* and *T. parvula* was also unique compared to that character in the other species analysed. Both species maintained a functional day/night cycle of stomatal aperture (Fig. 7), which, under water/salt stress, was only affected in amplitude, i.e. showing reduced opening during the day. The reduction of the daily flux was relatively lower in *Arabidopsis*, which had higher day-transpiration in the



absence of stress, a trait that is possibly distinctive of glycophytic species. Either a minor reduction of the daily transpiration or a loss of diurnal fluctuations was found in *L. virginicum* and *D. pinnata*. Despite the reduced transpiration flux, the relative water loss was much higher in *L. virginicum* compared to the other genotypes.

#### *Ion homeostasis*

The ability of plants to control cytoplasmic  $\text{Na}^+$  accumulation against vacuolar compartmentation is critical for determining salt tolerance in both glycophytes and halophytes (Hasegawa *et al.*, 2000; Munns, 2002; Parks *et al.*, 2002). However, the occurrence of a relative greater variation among halophytic respect to glycophytic species (Greenway and Munns, 1980; Yokoi *et al.*, 2002; Tester and Davenport, 2003) suggests that, in the former, additional salt tolerance effectors may exist (Volkov *et al.*, 2004; Kant *et al.*, 2006; Volkov and Amtmann, 2006). Minimizing bypass flow and other traits such as reduced transpiration have been proposed to contribute to the superior performance of halophytes under highly saline conditions (Flowers *et al.*, 1977, 1986; Yeo *et al.*, 1987; Lovelock and Ball, 2002). For instance, salt cress develops a double endodermis and it employs reduced transpiration [also observed in all halophytic species under assessment (Fig. 7)], with both characters contributing in restricting  $\text{Na}^+$  accumulation by reducing bypass flow (Inan *et al.*, 2004). Uncertainties about fundamental mechanisms of  $\text{Na}^+$  uptake/distribution/compartimentation within plants, as well as on  $\text{Na}^+/\text{K}^+$  selectivity, gradually become comprehensible by comparative analysis of *Arabidopsis* versus ARMS and/or other halophytes (Flowers and Colmer, 2008).  $\text{Na}^+$  influxes in halophytes are significantly lower than those found for *Arabidopsis* (Fig. 9). However,  $\text{Na}^+$  uptake in *T. salsuginea* seems to be mediated by a voltage-dependent channel similar to the glycophytic process (Demidchik and Maathuis, 2007). In addition, reduction in the expression of the SOS1  $\text{Na}^+/\text{H}^+$  transport system changed *Thellungiella* that normally can grow in seawater-strength sodium chloride solutions into a plant as sensitive to  $\text{Na}^+$  as *Arabidopsis* (Oh *et al.*, 2009) suggesting that halophytes and glycophytes share similar transporters and regulatory networks, but that different set points exist (Flowers and Colmer, 2008). One such set point seems to be basal gene expression strength and timing of expression in salt cress (Gong *et al.*, 2005; Oh *et al.*, 2009). Reduced  $\text{Na}^+$  flux has been confirmed in salt cress and found to exist in *T. parvula* (Fig. 10). However, behaviours distinguishing *T. salsuginea* and *T. parvula* regarding  $\text{K}^+$  transport and accumulation have been observed, possibly pointing towards several mechanisms for establishing ion homeostasis to cope with ion toxicity in halophytes (Volkov and Amtmann, 2006). Upon salinization, the larger  $\text{K}^+$  availability in *T. parvula* compared with both *A. thaliana* and *T. salsuginea* (Fig. 10) was correlated with a higher salinity tolerance (Figs 1–3). A representation summarizing the various parameters that have been recorded in this study is presented in Fig. 11.



**Fig. 11.** Representation summarizing the various parameters that have been recorded in this study. Different species are compared based on their relative ability to express pathways and phenotypes that support extremophile behaviour and success in adaptation. The species can be sorted in such a way that indicated *T. parvula* and *T. salsuginea* as the most successful species.

#### *Ranking abiotic stress responses: Thellungiella parvula as a new model species for comparative analyses of halophytes*

Several studies have established that the growth characteristics of salt cress identify the species as a halophyte (Gong *et al.*, 2005; M'rah *et al.*, 2006). Because of the ease of transformation, salt cress mutants with a loss or gain of tolerance are forthcoming and the genes responsible will eventually be identified. This will be significantly enhanced by the release of genomic sequences of salt cress and its close relative, *Thellungiella parvula*. At present, the genome sequences of both species have been determined and are in the final assembly phase (JGI webpage for salt cress; M Dassanayake, DH Oh, RA Bressan, JK Zhu, HJ Bohnert, personal communication, for *T. parvula*). EST sequence comparisons between *Arabidopsis* and promising

ARMS will reveal important similarities in the functional determinants of salt tolerance, similar to what has been already shown with *T. salsuginea* (<http://www.life.uiuc.edu/bohnert/projects/thel.html>). It has been pointed out that irrespective of high DNA sequence identity (90–95%) for the majority of transcripts between *Arabidopsis* and salt cress, there is lower conservation between genes that are known as salt-tolerance determinants in *Arabidopsis* (Inan *et al.*, 2004). For example, sequence variations that distinguish *Arabidopsis* from a range of halophytes in the C-terminal region of the SOS1 gene are much more pronounced than the variations in the N-terminus (see Supplementary Table S1 at *JXB* online). The N-terminus of AtSOS1 forms the transporter moiety of the Na<sup>+</sup>/H<sup>+</sup> antiporter protein, whereas the C-terminus is involved in, at present, largely unknown regulatory functions (Katiyar-Agarwal *et al.*, 2006; Olias *et al.*, 2009). More detailed comparisons with extremophile ARMS should reveal active functional sites within this C-terminus. In addition, genomic sequences of ecotypes within extremophile ARMS should allow the use of genome-wide association mapping and other genomic-based correlation studies. The information obtained from the study of extremophile ARMS will need to be supplemented with genomic studies of near extremophile wild relatives of crop species. This is especially important in light of the view that present-day halophytic land plants reestablished halophytism from glycophytic progenitors in parallel lineages (Flowers *et al.*, 1986).

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