Salt Cress. A Halophyte and Cryophyte Arabidopsis Relative Model System and Its Applicability to Molecular Genetic Analyses of Growth and Development of Extremophiles[1]

Günsu Inan, Quan Zhang, Pinghua Li, Zenglan Wang, Ziyi Cao, Hui Zhang, Changqing Zhang, Tanya M. Quist, S. Mark Goodwin, Jianhua Zhu, Huazhong Shi, Barbara Damsz, Tarif Charbaji, Qingqiu Gong, Shisong Ma, Mark Fredricksen, David W. Galbraith, Matthew A. Jenks, David Rhodes, Paul M. Hasegawa, Hans J. Bohnert, Robert J. Joly, Ray A. Bressan*, and Jian-Kang Zhu

Center for Plant Environmental Stress Physiology, Purdue University, West Lafayette, Indiana 47907–2010 (G.I., T.M.Q., S.M.G., J.Z., H.S., B.D., M.A.J., D.R., P.M.H., R.J.J., R.A.B.); Atomic Energy Commission, Damascus, Syria (T.C.); Department of Plant Biology, University of Illinois, Urbana, Illinois 61801 (Q.G., S.M., M.F., H.J.B.); Department of Plant Sciences, University of Arizona, Tucson, Arizona 85721 (C.Z., D.W.G., J.-K.Z.); and The Provincial Lab of Plant Stress, Shandong Normal University, Jinan 250014, People's Republic of China (Q.Z., P.L., Z.W., Z.C., H.Z.)

Salt cress (*Thellungiella halophila*) is a small winter annual crucifer with a short life cycle. It has a small genome (about 2 × Arabidopsis) with high sequence identity (average 92%) with Arabidopsis, and can be genetically transformed by the simple floral dip procedure. It is capable of copious seed production. Salt cress is an extremophile native to harsh environments and can reproduce after exposure to extreme salinity (500 mM NaCl) or cold to −15°C. It is a typical halophyte that accumulates NaCl at controlled rates and also dramatic levels of Pro (>150 mM) during exposure to high salinity. Stomata of salt cress are distributed on the leaf surface at higher density, but are less open than the stomata of Arabidopsis and respond to salt stress by closing more tightly. Leaves of salt cress are more succulent-like, have a second layer of palisade mesophyll cells, and are frequently shed during extreme salt stress. Roots of salt cress develop both an extra endodermis and cortex cell layer compared to Arabidopsis. Salt cress, although salt and cold tolerant, is not exceptionally tolerant of soil desiccation. We have isolated several ethyl methanesulfonate mutants of salt cress that have reduced salinity tolerance, which provide evidence that salt tolerance in this halophyte can be significantly affected by individual genetic loci. Analysis of salt cress expressed sequence tags provides evidence for the presence of paralogs, missing in the Arabidopsis genome, and for genes with abiotic stress-relevant functions. Hybridizations of salt cress RNA targets to an Arabidopsis whole-genome oligonucleotide array indicate that commonly stress-associated transcripts are expressed at a noticeably higher level in unstressed salt cress plants and are induced rapidly under stress. Efficient transformation of salt cress allows for simple gene exchange between Arabidopsis and salt cress. In addition, the generation of T-DNA-tagged mutant collections of salt cress, already in progress, will open the door to a new era of forward and reverse genetic studies of extremophile plant biology.

Salinity is a severe and increasing constraint on the productivity of agricultural crops. High concentrations of salts in the soil have a strong inhibitory effect on the growth and harvestable yield of all crop species. Secondary salinization significantly impairs crop production on at least 20% of irrigated land worldwide (Ghassemi et al., 1995), and irrigated agriculture contributes more than 30% of global agricultural production (Hillel, 2000). Salinization of arable land arising from poor water management has led to the decline of past civilizations, and it threatens the long-term sustainability of many current large-scale irrigation systems, especially those in Asia (Sharma and Goyal, 2003). Soil salinity almost always originates from previous exposure to seawater (Flowers et al., 1986). Although it is believed that, for most of the Earth's history, the salt level of the oceans was much lower than at present (Serrano et al., 1997), all plant species that inhabit the seas, as well as a phylogenetically diverse groups of land plants, are capable of growth and reproduction at salinity levels near or above those found in the seas at present (Serrano et al., 1997). This strongly supports the existence of a genetic basis for high-salinity tolerance within both sea and land plants. The wide distribution of salt-tolerant plants

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* Corresponding author; e-mail bressan@hort.purdue.edu; fax 765–494–0391.

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(halophytes) among diverse plant families (Flowers et al., 1986; Gorham, 1992) suggests either a polygenic ancient origin of salt tolerance or its independent origin via a multitude of possible avenues (Flowers et al., 1986). Nevertheless, some remarkable similarities in the characteristics of halophytes have been observed in the numerous studies aimed at the elucidation of the physiological and underlying genetic bases of their salinity tolerance.

Although some prokaryotic species produce enzymes and general metabolic machinery capable of functioning in the presence of very high levels of Na^+, no halophile eukaryotic plants are known to have this capability (Yeo, 1998). This does not exclude the possibility of individual enzymes, or entire metabolic pathways, having evolved to function in the presence of high sodium in halophytes, but very little attention has been devoted to the analysis of such possible mechanisms. Present knowledge compels the assumption that avoiding contact of the metabolic machinery with high levels of Na^+ is a prerequisite of halophytism. Although halophytes typically are classified as either ion excluders or accumulators, it is now recognized that the ability to tightly regulate the net rate of uptake of Na^+ and Cl^- ions and their distribution within the plant, rather than to control the level of total accumulation, is a fundamental attribute of halophytes, which is insufficient or lacking in glycophytes (Hasegawa et al., 2000). Controlled uptake of NaCl is also critical to the ability of halophytes to utilize external Na^+ and Cl^- for osmotic adjustment without suffering the consequences of ion toxicity.

The tightly controlled uptake (i.e. net influx/efflux) of Na^+ and Cl^- ions, even at very high external concentrations, is closely coordinated with growth in halophytes, although controversy persists as to whether growth is limited by the ability to rapidly accumulate ions or whether the growth rate determines the physiology of salt accumulation (Flowers et al., 1986; Munns, 2002). Establishing the cause and effect in this correlation has to consider that halophytes sequester accumulating Na^+ and Cl^- into vacuoles, thus avoiding toxic effects to sensitive metabolism in the cytosol. Thus high rates of Na^+ ion uptake into cells, in the absence of an efficient compartmentalization mechanism, will result in a negative correlation between total cell uptake and growth. Conversely, high rates of Na^+ uptake, in conjunction with its rapid transport to the vacuole, will lead to a strong positive correlation between ion accumulation and growth rate. In this case, Na^+ could be viewed as a positive (or even required) component of cell growth (Flowers et al., 1977, 1986; Glenn et al., 1999). It can be concluded that plants that are capable of both tightly controlled cell uptake and high rates of vacuolar sequestration are able to maintain fast growth in the presence of high salinity, presumably by harnessing the osmotic potential of accumulated ions.

In spite of solving the problem of cytoplasmic ion toxicity, the vacuolar sequestration of ions by halophytes creates an osmotic imbalance with the cytoplasm. One mode of adjustment could be a shift in the relative volumes occupied by cytosol and vacuole in the cell (see Binzel et al., 1988). More importantly, it is commonly observed that concomitant with controlled ion uptake and sequestration of toxic ions into the vacuole, halophytes, upon exposure to external NaCl, also synthesize and accumulate large amounts of metabolites that are compatible with and even helpful in stabilizing cytosolic metabolism (Flowers et al., 1986). The compatible osmolytes include a variety of metabolites that are at least temporarily used as end products and include polyols, cyclitols, quaternary ammonium compounds, amino acids, and sugars (Greenway and Munns, 1980; Hasegawa et al., 2000). These metabolites are invariably connected to major fluxes of carbon and nitrogen during exposure to saline stress.

Regulation of water flux through the plant has emerged as an important component of salinity tolerance in halophytes. Halophytes exhibit reduced stomatal conductance compared to glycophytes, and transpiration is often further decreased with increased exposure to salinity (Flowers et al., 1986; Serrano et al., 1997). Of the four critical control steps in membrane transport of ions necessary for salt tolerance discussed by Flowers et al. (1986), both the entry of ions into the xylem from root cortical cells and their subsequent exit from xylem into shoot cells are greatly influenced by the rate of transpiration. Thus, the salt load of the shoot (leaves) can be dramatically influenced by stomatal function (Lovelock and Ball, 2002). This can be readily confirmed by the large increase in tolerance of glycophytes observed when their transpiration is inhibited.

Despite considerable efforts during the past several decades to understand the underlying genetic bases of the physiology of salinity tolerance, little progress was made until the introduction of Arabidopsis as a genetic model system to study salinity tolerance (Zhu, 2000, 2001a, 2002). In view of the fact that Arabidopsis is a glycophyte that does not exhibit salinity tolerance even approaching that of halophytes, its utilization in salinity tolerance studies initially may have seemed inappropriate. However, forward genetic studies using the Arabidopsis model have yielded essential new knowledge about the genetic bases of salinity tolerance (Zhu, 2002). Unfortunately, the genetically intractable nature of all halophytes studied to date has precluded their use in comparable efficient genetic analyses. To our knowledge, *Mesembryanthemum crystallinum* is the only halophyte where a systematic attempt has been made to isolate salt-sensitive mutants and the relevant genes involved (Adams et al., 1998). However, without the efficient molecular genetic tools that are available for Arabidopsis, there is little chance that the altered genes responsible for a loss of tolerance in these mutants will be identified. Therefore, functional analysis of the special physiology and biochemistry of halophytes that imparts their exceptional degree of salt tolerance will require the utilization of a halophytic

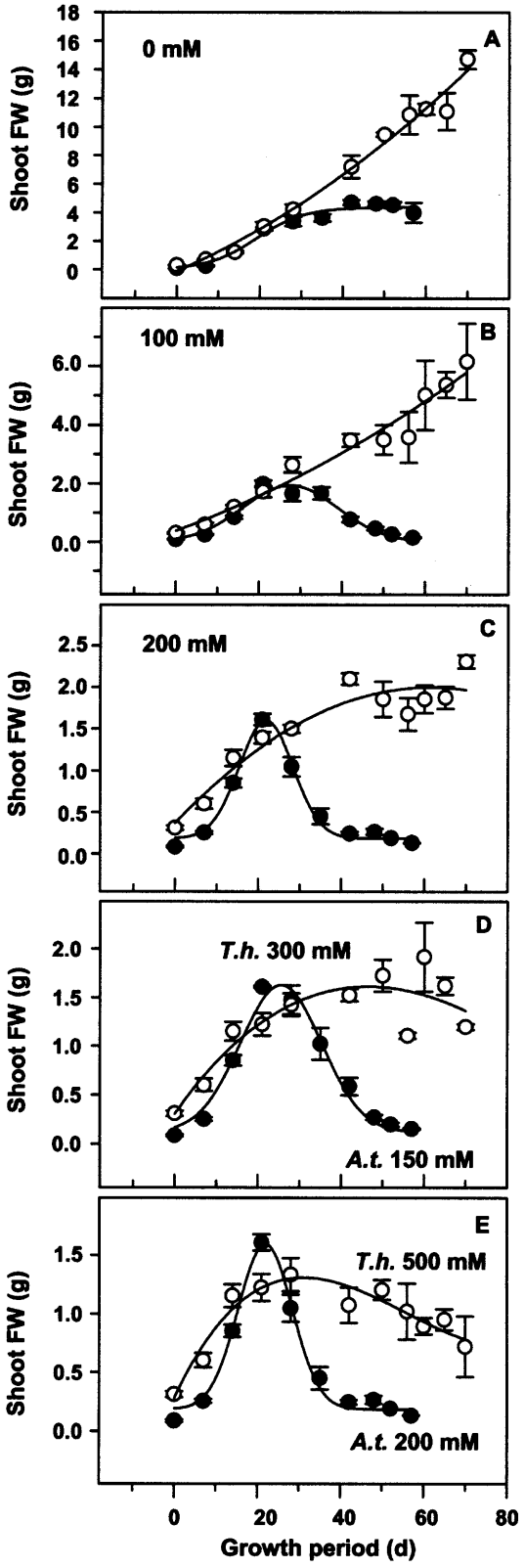


Figure 1. Effect of NaCl on shoot FW of salt cress and Arabidopsis (Col *gl1*). Plants were grown from seeds in Turface, and NaCl was increased incrementally in the irrigation water every 7 d to final concentrations of 100, 150, and 200 mM for Arabidopsis and 100, 200, 300, and 500 mM for salt cress. Shoots were harvested every 7 d through day 42, after which time the sampling interval was reduced to 4 to 6 d. A to C, Comparisons of the two species at 0, 100, and 200 mM, respectively. D and E, Effects of unequal treatment levels (higher in salt cress and lower in Arabidopsis) because concentrations above 200 mM were lethal in Arabidopsis. White circles, salt cress (*T.h.*); black circles, Arabidopsis (*A.t.*). Values are means ± SE; $n = 8$.

genetic model system that incorporates the most important features of the Arabidopsis model. We have recently reported the existence of such a model halophyte, which is a close relative of Arabidopsis in the genus Thellungiella (Bressan et al., 2001; Teusink et al., 2002). *Thellungiella halophila*, previously classified as *Arabidopsis halophila*, recently has been reclassified as *Thellungiella salsuginea* (Al-Shehbaz et al., 1999), which now can be considered synonymous with *Thellungiella halophila* (salt cress). The life cycle of salt cress is very similar to that of Arabidopsis, and it shares important morphological and phenological attributes with Arabidopsis that are necessary for rapid efficient genetic analyses (Bressan et al., 2001).

Successful adaptation to salinity involves four interacting basic signal perception-response systems: ion homeostasis, osmotic adjustment, injury avoidance, and growth adjustment (Zhu, 2001a). We demonstrate that salt cress displays extreme capability in all of these response systems. We report several specific developmental, physiological, biochemical, and genetic characteristics of salt cress that establish this small, short-life-cycle crucifer as a true extremophyte for the specific traits of extreme salinity and cold tolerance. Although exceptional cold tolerance could be related to halophytism through injury avoidance mechanisms, cold tolerance of halophytes has not been seriously examined. Salt cress may now offer this opportunity as it possesses growth and many other characteristics that are typical of halophytes. More than any other reported halophyte, salt cress offers simplicity and efficiency for genetic analyses. It represents an outstanding case of a trait-specific genetic system that also provides the needed powerful molecular genetic tools developed for the Arabidopsis system.

RESULTS

Salt Cress Morphology and Life Cycle

Salt cress shares many important features with Arabidopsis (Bressan et al., 2001). It is a small-stature rosette plant about the size of Arabidopsis that can complete its life cycle in 6 to 8 weeks. Similar to Arabidopsis, germination rate and frequency are increased by a 4°C stratification treatment of 3 to 10 d, and flowering is accelerated by a vernalization period of about 3 weeks. Without vernalization, salt cress plants will eventually and sporadically generate flowers only after 10 to 12 months. Growth of young seedlings is somewhat slower compared to Arabidopsis, but eventually they display very similar growth rates without NaCl (Figs. 1 and 2). We have obtained

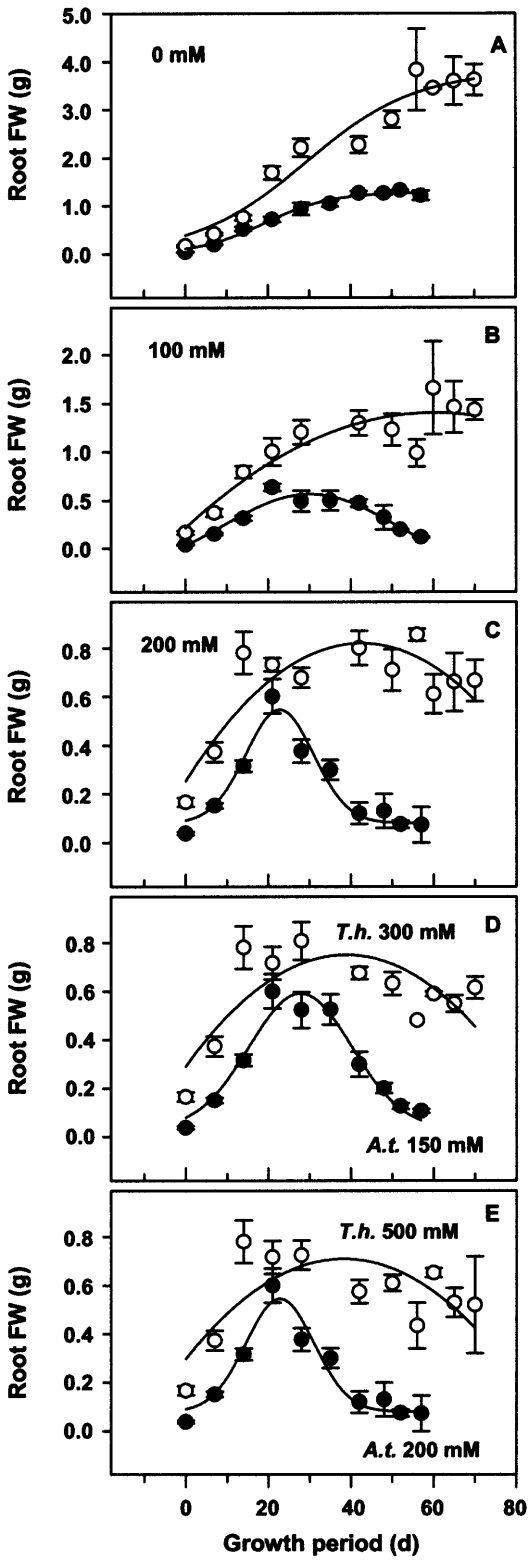


Figure 2. Effect of NaCl on root FW of salt cress and Arabidopsis (Col *gl1*). Plants were grown from seeds in Turface, and NaCl was increased incrementally in the irrigation water every 7 d to final concentrations of 100, 150, and 200 mM for Arabidopsis and 100, 200, 300, and 500 mM for salt cress. Roots were harvested every 7 d through day 42, after which time the sampling interval was reduced to 4 to 6 d. A to C, Comparisons of the two species at 0, 100, and 200 mM, respectively. D and E, Effects of unequal treatment levels (higher in salt cress and lower in Arabidopsis) because concentrations above 200 mM were lethal in Arabidopsis. White circles, salt cress (*T.h.*); black circles, Arabidopsis (*A.t.*). Values are means ± SE; $n = 8$.

ethyl methanesulfonate (EMS) mutants of salt cress that lack the vernalization requirement for flowering and can be used to shorten the life cycle even further (J.K. Zhu, unpublished data). Flower and silique structures are very similar to Arabidopsis (Bressan et al., 2001; Zhu, 2001a), and copious amounts of seeds (approximately. 6,000–8,000 per plant) are produced on multiple flower stalks that mature less synchronously and over a longer time period than those of Arabidopsis.

Growth in NaCl

Salt cress plants exhibit extreme tolerance to either NaCl shock exposure or to gradually increasing levels of NaCl (Figs. 1–3), but not to increasing soil desiccation (data not shown). Both root growth and shoot growth continue at NaCl levels up to 500 mM (essentially as in seawater; Figs. 1–3). In contrast, Arabidopsis seedlings continue growth in the presence of up to only 200 mM NaCl for approximately 20 d, but then fresh weight (FW) declines precipitously (Figs. 1 and 2) and the plants die. The FW decline of salt cress plants measured after longer exposures to high levels of NaCl is due principally to the loss of older senescent leaves, a pattern observed for many halophytes (Flowers et al., 1986). Salt cress plants eventually cease net FW gain, although new leaves and reproductive structures continue to form even at 500 mM NaCl. These plants are able to survive this stress for several months during which they continue to produce viable seeds.

Salt Cress Plants Preadapted to High NaCl Levels Survive Sudden Osmotic Downshock

The cultured cells of typical glycophytic plants can adapt and grow in high levels of NaCl (up to 500 mM), but only if the NaCl concentration is increased gradually. Likewise, they cannot survive a sudden return to an environment with much less NaCl (i.e. more positive osmotic potential; Bressan et al., 1990). Salt cress, however, can survive both a NaCl shock exposure (Bressan et al., 2001; Zhu, 2001a; Figs. 1 and 2) and a sudden osmotic downshock from 500 mM NaCl to 0 mM NaCl (Fig. 3). Upon removal of NaCl from the nutrient solution, salt cress plants resumed rapid growth and exhibited a dramatic increase in FW (Fig. 3). This remarkable ability to survive and grow after both sudden increases and decreases in the ion content and osmotic potential of the nutrient solution strongly suggests that not only are salt cress plants able to regulate ion fluxes in tissues, but also they are capable of very strict control of ion movements into and out

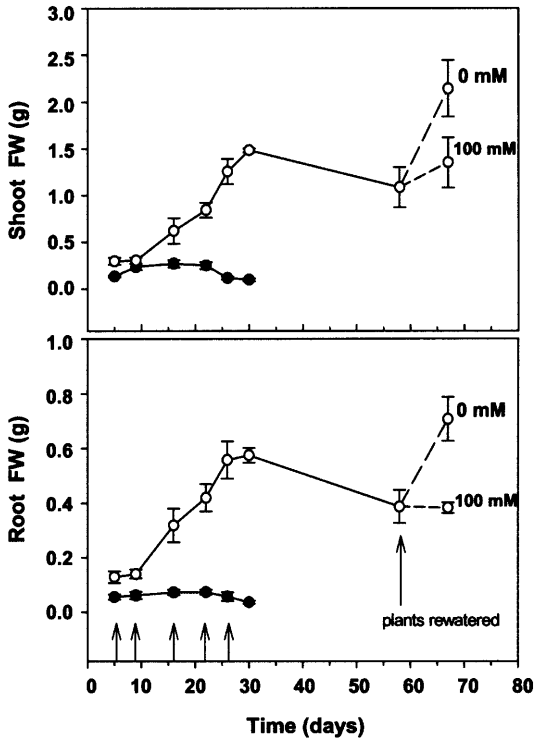


Figure 3. Effect of increasing concentration of NaCl on shoot and root FW of salt cress and Arabidopsis (Col *gl1*). Three-week-old seedlings grown in Turface were irrigated with 75 mM NaCl for 5 d, and salt concentration was then gradually increased to 150, 200, 300, 400, and 500 mM on days 5, 9, 16, 22, and 26, respectively, as denoted by vertical arrows on the *x* axis. Salt cress plants were irrigated every other day with 500 mM NaCl from day 30 to 58. After sampling on day 58, the remaining plants were rewatered with either 0 or 100 mM, and growth was assessed again after 9 d. Arabidopsis did not survive beyond day 30. White circles, salt cress; black circles, Arabidopsis. Values are means ± SE; $n = 8$.

of their cells, across both tonoplast and plasma membrane. The mechanisms involved apparently are sufficient to prevent damage and rupture of cell membranes and other injury responses that would inevitably follow sudden changes in the external osmotic environment. This capability may be manifested as tolerance to cold shock (see below) in a way that may be mechanistically related to osmotic shock (Zhu, 2001b).

Ion Uptake in Salt Cress Is Highly Regulated during Salt Exposure

Consistent with the characteristics of growth and survival of salt cress during both ion stress upshock and downshock experiments (Figs. 1–3), the accumulation of Na^+ ions in leaves of salt cress was much lower than that observed in Arabidopsis at external concentrations between 0 and 200 mM NaCl (Fig. 4). Sodium accumulation increased sharply in Arabidopsis leaves until the plants died, but leaf Na^+ content of salt cress increased less at external concentrations between 100 and 500 mM NaCl. Large reductions in the tissue content of K^+ also occurred in Arabidopsis as Na^+ ions accumulated (Fig. 4). In contrast, salt cress exhibited only modest reductions in K^+ levels, even after exposure to very high levels of NaCl (Fig. 4), a response characteristic of many halophytes.

Pro Is the Major Compatible Osmolyte That Accumulates to Counterbalance Ion Accumulation in Salt Cress

Several potential compatible osmolytes were assayed in plants throughout a 70-d exposure to a range of NaCl concentrations. Although small amounts of choline and trigonelline were found in both Arabidopsis and salt cress, no increase in quaternary ammonium compounds could be detected in either species, and only a modest accumulation of sugars was found (Fig. 5B). However, the amino acid Pro accumulated to very high levels in leaves of salt cress after salt treatment (Fig. 5A). No significant accumulation of the other 19 natural amino acids could be measured in response to NaCl (data not shown).

Germination of Salt Cress Seeds Is Hypersensitive to NaCl

Exposure to NaCl greatly reduced the ability of salt cress seeds to germinate after a 7-d period of

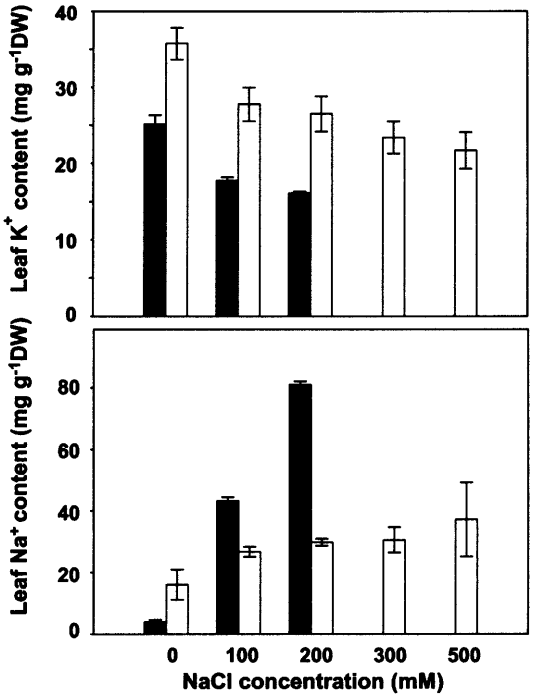


Figure 4. Effect of NaCl on Na^+ and K^+ content in leaves of salt cress and Arabidopsis. Three-week-old plants were grown in Turface and salinized incrementally, every 7 d, to final concentrations of 0, 100, 200, 300, and 500 mM NaCl. For salt cress, concentration was incremented at 100 mM intervals, while 50 mM increments were used for Arabidopsis. Arabidopsis did not survive NaCl concentrations higher than 200 mM. Na^+ and K^+ content were measured 28 d after the onset of salt treatment. White bars, salt cress; black bars, Arabidopsis. Values are means ± SE; $n = 9$.

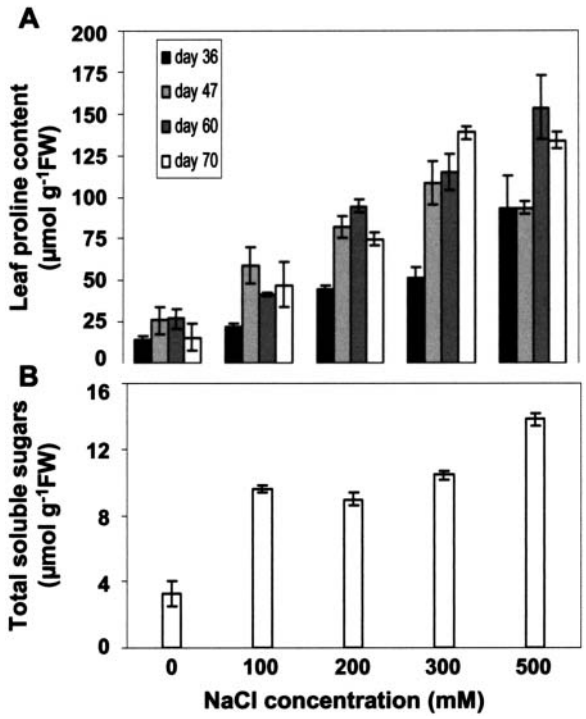


Figure 5. Pro and total soluble sugar accumulation of salt cress plants subjected to NaCl stress. A, Three-week-old plants were grown in Turface and salinized incrementally, every 7 d, to final concentrations of 0, 100, 200, 300, and 500 mM NaCl. Leaf tissue was sampled for Pro determination on days 36, 47, 60, and 70.Values are means ± SE, n=3. B, Four-week-old plants were grown in soil and treated with four levels of NaCl for 3 weeks. Total soluble sugar accumulation was measured by the anthrone method. Values are means ± SE, n = 3.

imbibition, and this inhibition was greater than that observed with seeds of Arabidopsis (Fig. 6A). Sensitivity of germination to elevated salinity has been reported for seeds of other halophytes (Flowers et al., 1986).

Germination of salt cress seeds in the absence of salt is typically close to 100%, but germination rate is not uniform. A portion of the seeds will germinate immediately, much the same as in Arabidopsis, whereas germination of other subpopulations is spaced out (data not shown). This behavior is independent of the presence of NaCl, and spacing of germination extends to 3 to 4 months after sowing, with approximately one-third of the total seeds germinating in each of three waves. Delayed germination is known for other species as well, apparently ensuring maximum survival.

Although Arabidopsis seed germination is quite sensitive to direct exposure to NaCl (Fig. 6A), germination inhibition caused by treatment of seeds with very high levels of NaCl can be largely reversed by rescue of the seeds to a medium without NaCl (Fig. 6B). Interestingly, salt cress seeds are less able to be rescued from NaCl treatment, indicating that seeds of salt cress may be injured by NaCl at the embryo and early developmental stages after breaking seed dormancy (Fig. 6B), perhaps because of constitutive or rapid deployment of ion transporters that facilitate Na^+ influx into the embryo. It is more likely, however, that the initial exposure to NaCl for 7 d during seed imbibition resulted in an enhanced dormancy state, delaying germination well beyond the time allotted in

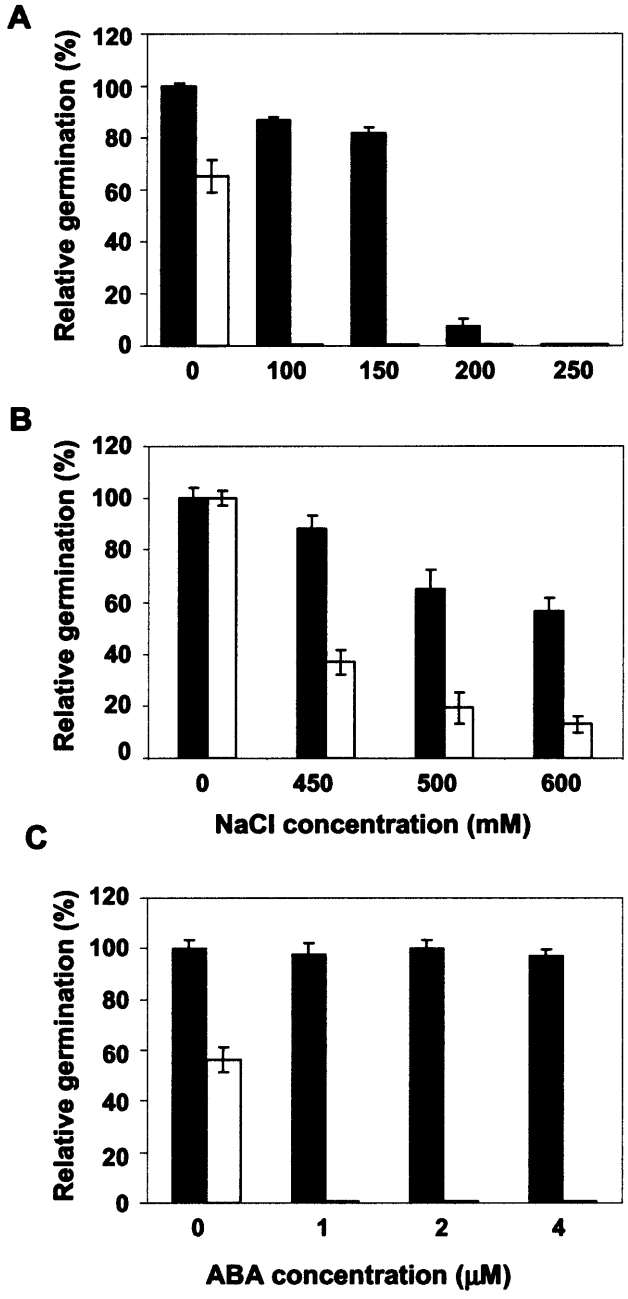


Figure 6. The effect of NaCl on germination of salt cress and Arabidopsis (Col *gl1*). A, Seeds were sown on agar plates containing MS medium or MS medium supplemented with NaCl. Germination was recorded 7 d after sowing. B, Seeds were sown on agar plates containing MS medium supplemented with NaCl and rescued to MS medium without salt after 8 d. Germination was measured following an additional 3 d and calculated as a percentage relative to that on MS medium. C, Seeds were sown on agar plates containing MS medium supplemented with ABA. Germination was recorded 10 d after sowing. White bars, salt cress; black bars, Arabidopsis. Values are means ± SE; n = 3.

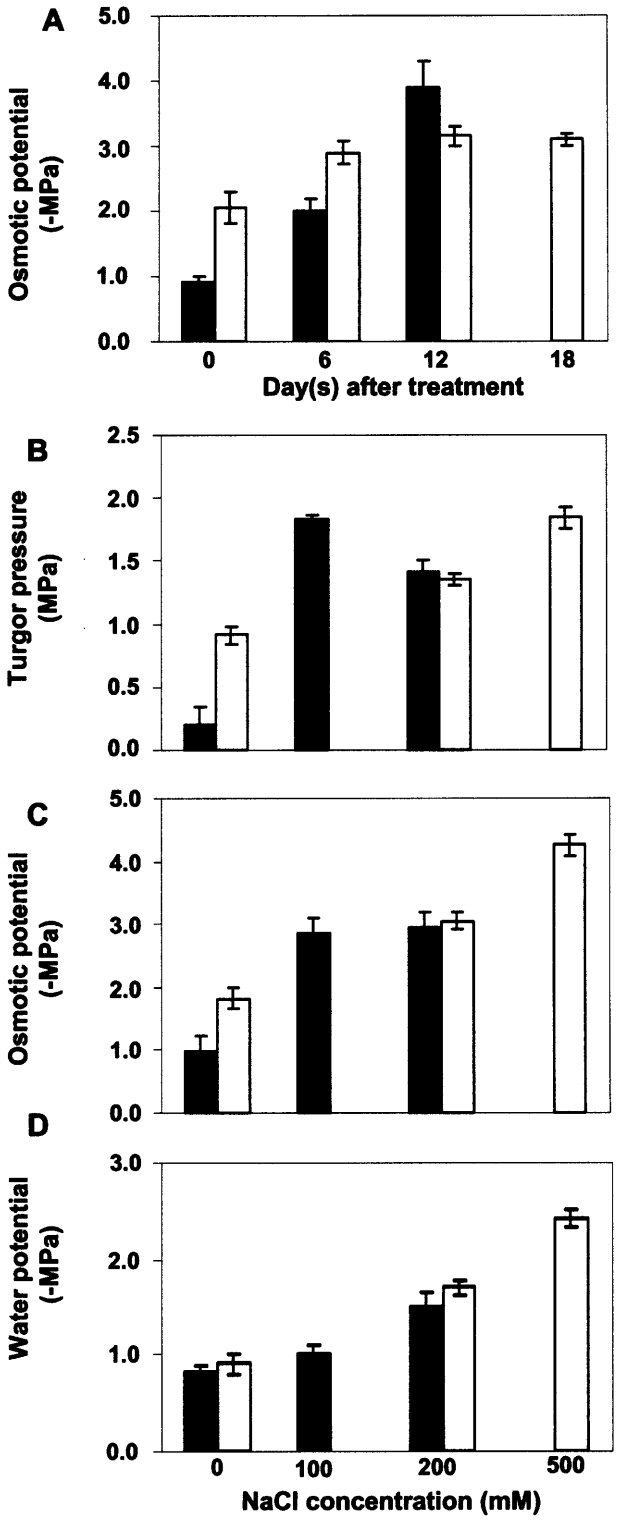


Figure 7. Tissue water relations of salt cress and Arabidopsis (Col *gl1*) plants subjected to NaCl stress. Plants were grown in Turface and irrigated with four levels of NaCl for 18 d. Measurements were made every 6 d and data were combined over all sample dates. Responses of salt cress to 100 mM NaCl were not determined. A, Leaf osmotic potential measured on expressed sap of frozen and thawed leaf samples of plants irrigated with 200 mM NaCl for 18 d. B, Leaf turgor pressure was estimated as the difference between water potential and osmotic potential. C, Leaf osmotic potential measured as in A. D, Leaf water potential was measured in single leaves with a Scholander-type pressure chamber. White bars, salt cress; black bars, Arabidopsis. Values are means ± SE; $n = 9$.

our experiments, since salt cress seed germination is hypersensitive to abscisic acid (ABA; Fig. 6C). Nevertheless, this apparent enhanced sensitivity to NaCl is short-lived in salt cress seeds since seedlings begin to show greater salt tolerance (growth), compared to Arabidopsis, as early as 10 d after germination (data not shown).

Water Relations during Salt Stress Reflect the Ability to Control Ion Uptake

In the absence of NaCl, salt cress plants exhibited a constitutive osmotic potential significantly more negative than that measured in Arabidopsis (Fig. 7, A and C). Further, the osmotic potential of Arabidopsis plants declined rapidly to very low values (400% decrease within 12 d) upon treatment with 200 mM NaCl, followed soon after by death. In contrast, the osmotic potential of salt cress declined by only approximately 38% during the initial 6 d of exposure, but thereafter remained nearly constant throughout the treatment (Fig. 7A). When salt cress plants were exposed to concentrations as high as 500 mM NaCl, they were capable of lowering their leaf osmotic potential to below −4.0 MPa, a level sufficient to maintain turgor pressure (Figs. 7, B–D). Thus, all of the water relation adjustments that were observed with salt cress during stress were consistent with controlled accumulation and sequestration of NaCl.

Stomatal Control of Salt Cress Is Typical of Halophytes

Halophytes typically exhibit reduced transpiration rates compared to glycophytes (Lovelock and Ball, 2002). The decreased stomatal apertures of halophytes prevent excessive water loss and, more importantly, reduce the movement of ions into the shoots during salt exposure (Lovelock and Ball, 2002). Salt cress had much lower transpiration rates during the day and almost no detectable water loss at night, compared to Arabidopsis (Fig. 8B). These gravimetric assessments of water loss were confirmed by stomatal conductance measurements using a gas-exchange system (Fig. 8A) and are consistent with numerous reports that halophytes exhibit decreased stomatal conductance following salt exposure (e.g. Lovelock and Ball, 2002). These results, considered in conjunction with the FW gains measured during NaCl stress (Figs. 1 and 2), suggest that salt cress, like other halophytes, benefits from a higher water use efficiency as a consequence of its much lower transpiration rate, especially during periods of salt exposure (Fischer and Turner, 1978). This must result from a nonproportional decline of

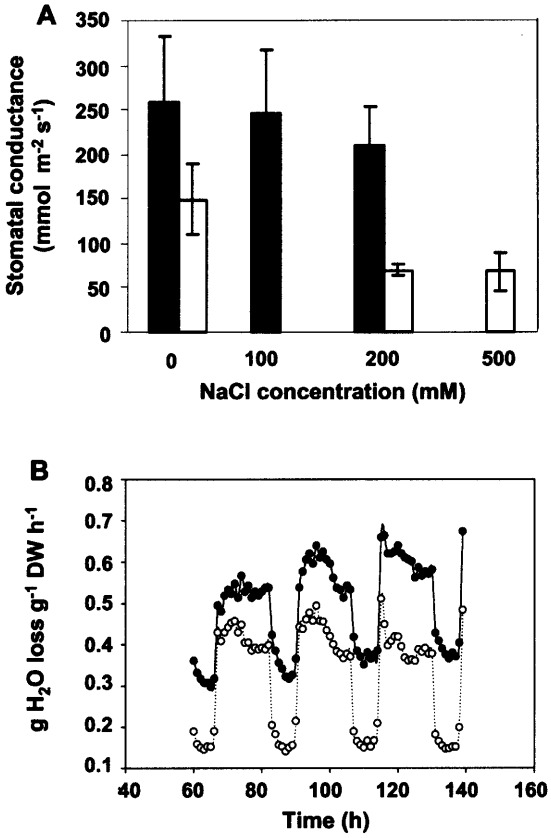


Figure 8. Stomatal conductance and diurnal whole-plant transpiration in salt cress and Arabidopsis (Col *gl1*). A, Plants were grown in Turface and irrigated with NaCl for 18 d. Stomatal conductance was measured with a PP Systems CIRAS-1 portable photosynthesis system every 6 d, and data are combined over all sample dates. Responses of salt cress to 100 mM NaCl were not determined. White bars, salt cress; black bars, Arabidopsis. Values are means ± SE; $n = 9$. B, Four-week-old Arabidopsis (Col-0) and equivalently sized salt cress seedlings grown under long-day conditions with cool-white fluorescent lighting were used for measurements of whole-plant water loss. Plants were grown singly in 9-cm pots, which were sealed in plastic wrap and placed on electronic balances. Weight was determined every 30 min for 7 d. Values are means of 8 and 16 plants for Arabidopsis and salt cress, respectively. White circles, salt cress; black circles, Arabidopsis.

CO_2 fixation with decreased water loss at low stomatal apertures.

Morphological Characteristics of Salt Cress Related to Halophytism

Several halophyte species show early development of Casparian strips on endodermal cell walls or even develop a second layer of endodermis (Flowers et al., 1986). Roots of salt cress also develop a second layer of endodermis and, at comparable stages of development to Arabidopsis (Dolan et al., 1993), also possess an additional cortex layer (Fig. 9C). These features may assist in the restriction of ion movement through the vasculature to xylem elements in the root and subsequently to shoot tissues. Thus stomatal behavior and root morphology of salt cress appear to act in conjunction to decrease the rate of ion flux into shoots. This is clearly reflected in the ion accumulation measurements shown in Figure 4, where salt cress accumulated Na^+ ions more gradually than did Arabidopsis.

Unlike Arabidopsis leaf morphology (Teffler and Poethig, 1994), salt cress displays succulent-like leaves after salt exposure, measured as FW to dry-weight (DW) ratio (Fig. 10). The development of a second layer of leaf palisade cells (Fig. 9, A and B) may contribute to this and also affect the rate of water loss from leaves. In addition, the stomatal density on salt cress leaves is twice that of Arabidopsis ($n = 9$; $P < 0.001$), although the stomatal index is nearly the same ($n = 9$; $P = 0.167$; Fig. 11; Table I). This may allow more efficient distribution of CO_2 to photosynthetic mesophyll cells at low stomatal apertures.

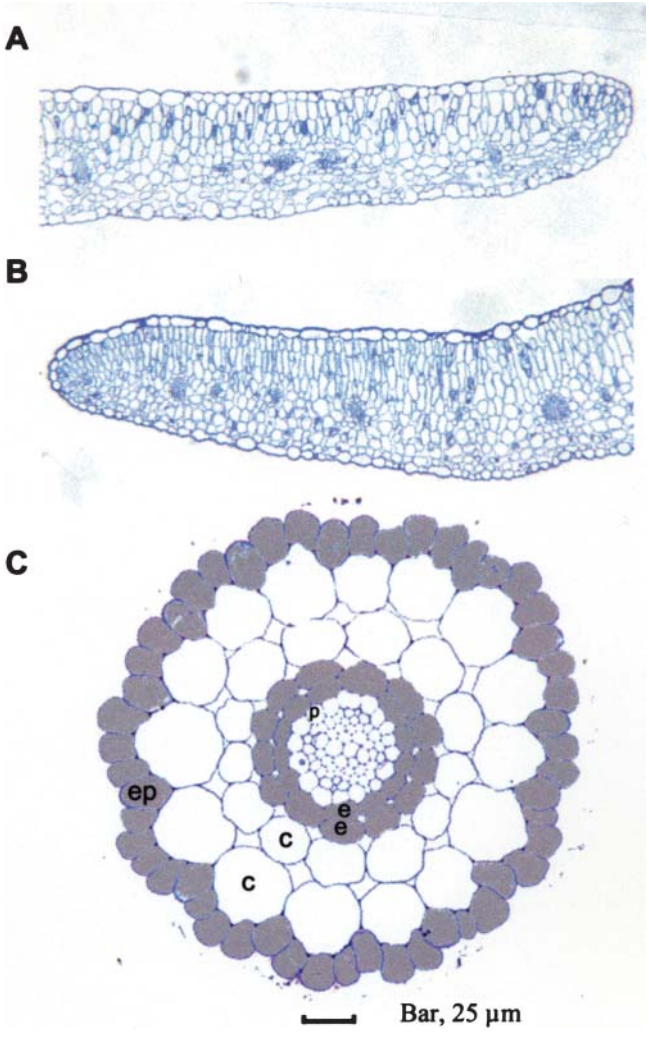


Figure 9. Cross-sectional leaf and root anatomy of salt cress. A, First fully expanded leaf from a 3-month-old plant. B, Mature fully expanded leaf, sampled from a 3-month-old plant that had been treated with 500 mM NaCl for 40 d. C, Root cross-section taken approximately 1 mm from the root tip. ep, Epidermis; c, cortex; e, endodermis; p, pericycle.

Cold Tolerance of Salt Cress

Plants of salt cress and Arabidopsis were acclimated at 4°C for 1 week and subsequently brought to −15°C. After 24 h at −15°C, Arabidopsis plants are completely killed, since they could not recover from this treatment when returned to the greenhouse. However, salt cress plants survived with a moderate amount of injury (Fig. 12). The shoot apex and young expanding leaves of salt cress were always more tolerant of subfreezing temperatures than mature leaves. The high level of freezing tolerance of salt cress could be related to its ability to survive salt shock (Figs. 1 and 2), since ability to survive both salt and cold shock likely involves an enhanced capacity to control deleterious injury responses (Zhu, 2001a).

Molecular Genetic Characteristics of Salt Cress

Although physiological and biochemical research on halophytes has been carried out extensively for decades (Flowers et al., 1977, 1986; Greenway and Munns, 1980; Yeo, 1998; Hasegawa et al., 2000; Zhu, 2001a, 2002; Xiong and Zhu, 2002), very little work on the genetic bases of tolerance has been reported. In very few instances have Mendelian loci controlling salt tolerance been identified in even moderately salt-tolerant plants (Epstein et al., 1980; Dubcovsky et al., 1995; Zhong and Dvořák, 1995), and only recently have mutations affecting the salt tolerance of halophytes been reported (Adams et al., 1998; Bohnert and Cushman, 2001), owing to the fact that there has been no genetically tractable halophyte model system available. As we report here, salt cress has several molecular genetic features that we believe will fundamentally change this.

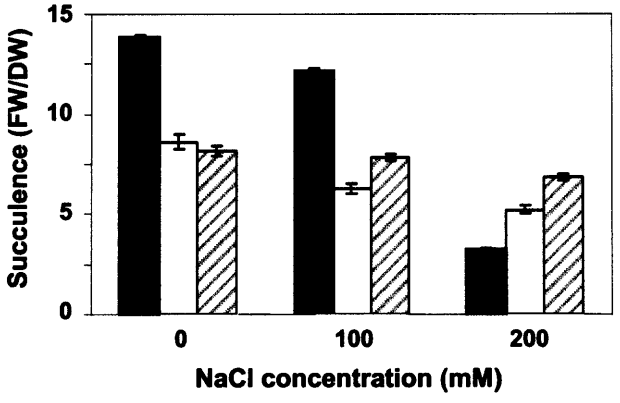


Figure 10. Effect of NaCl on shoot succulence of salt cress and Arabidopsis (Col *gl1*). NaCl was increased in the irrigation water in 50 mM increments every 7 d to final concentrations of 100 and 200 mM. NaCl treatment continued up to day 70, at which time plants were rewatered with 0 mM NaCl for an additional 19 d. FW to DW ratios were measured on day 42 in Arabidopsis (black bars) and salt cress (white bars) and on day 89 in salt cress (hatched bars); no Arabidopsis plants survived beyond 58 d. Values are mean ± SE; $n = 8$.

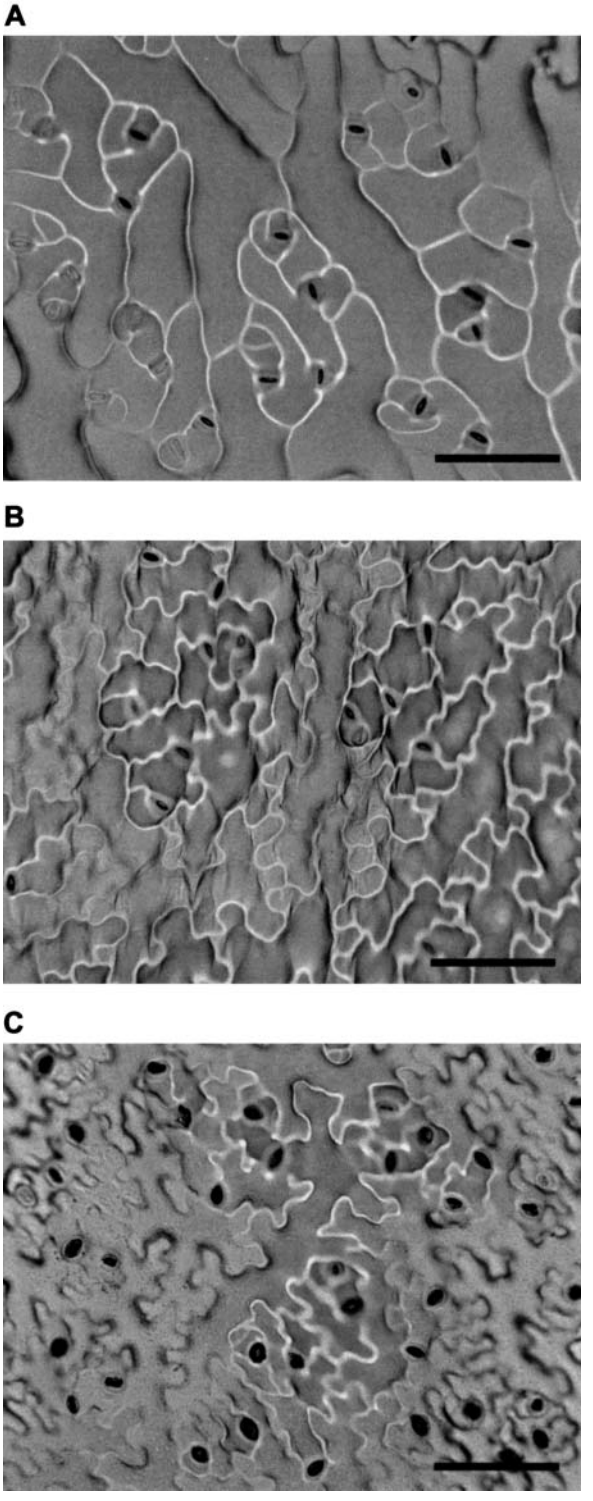


Figure 11. Bright-field light microscopic images of adaxial leaf surfaces of Arabidopsis and salt cress. Images are from leaf surface imprints obtained using cyanoacrylate adhesive. Scale bar is 100 μm. A, Arabidopsis, rosette leaf; B, salt cress, cauline leaf; C, salt cress, rosette leaf.

Table I. *Arabidopsis and salt cress plant stomatal and pavement cell densities (stomata or pavement cells per mm^2) and stomatal index of adult-phase leaf blades*

Values are means ± SD, $n = 9$.

	Arabidopsis	Salt Cress	
	Adaxial Rosette Leaf	Adaxial Rosette Leaf	Adaxial Cauline Leaf
Stomatal density	101.3 ± 11.3	203.3 ± 40.6[a]	114.3 ± 31.3
Pavement cell	243.2 ± 18.4	500.6 ± 151.4[a]	295.1 ± 102.3
Stomatal index	29.4 ± 1.0	29.5 ± 3.56	28.3 ± 3.0

[a]Significantly different from Arabidopsis ($P < 0.001$; Student's t test).

Gene Transfer in Salt Cress

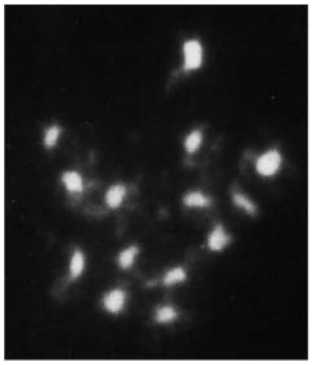
Salt cress has a small genome about twice the size of Arabidopsis, organized into seven chromosomes (Fig. 13; Bressan et al., 2001). Even though it cannot be crossed with Arabidopsis, gene exchange can be achieved by efficient transformation of salt cress with Arabidopsis genes and vice versa. An efficient salt cress transformation system will also assist the future use of salt cress for the discovery of genes that are important to extreme salt and cold tolerance. Using the floral dip procedure (Bent, 2000), transformation of salt cress can be as high as 1% (Bressan et al., 2001). However, it is usually achieved at frequencies between 0.1 and 0.3%. Frequencies of transformation were comparable with the two vectors used, pSK1015 (Weigel et al., 2000) and pE1829, based on detection of insertions by PCR amplification of the bialaphos marker and growth on selective (bialaphos) medium. Insertion sites into the genome, which were tested in nine randomly chosen transformed plants, indicated independent insertion events (data not shown).

Sequence Identity of Arabidopsis and Salt Cress Expressed Sequence Tags

In pilot experiments, expressed sequence tag (EST) sequences have been obtained from cDNA libraries from salt cress plants that had been stressed by the addition of 250 mM NaCl (24 h; http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?CMD=search&DB=nucleotide; query: "Thellungiella"). An analysis of approximately 1,600 sequences from cDNA libraries, generated without subtraction to obtain an abundance profile, indicated an overall similar profile to that of Arabidopsis, yet with some important differences. An annotation of these sequences can be found at http://www.life.uiuc.edu/bohnert/projects/thel.html. Next to the category of functionally unknown transcripts (46%, including transcripts where no clear categorization is possible), the rescue and defense category includes the most transcripts (12%), followed by the metabolism (10%) and energy (7%) categories, whereas transcripts in other categories are at lower abundance. One aspect is that most transcripts for well-known housekeeping genes in photosynthesis and basal metabolism show scores in the range of 90% to 95% nucleotide sequence identity, and high E values indicated their orthologous nature with respect to Arabidopsis genes. Other transcripts, many in categories related to stress responses, show significantly lower identity scores and deviate on the nucleotide level substantially. Indicated in Table II are transcripts related to Arabidopsis genes that are apparent paralogs of known genes in Arabidopsis. These paralogs, absent from Arabidopsis genome sequence, show amino acid identities in the range of 42% to 70% compared to the corresponding Arabidopsis genes. The table presents salt cress paralogs for the Arabidopsis cold-, drought-, and salt-inducible genes ERD10, COR47, KIN1, and KIN2, and several other functionally unknown sequences. Some of these novel

Figure 12. Cold tolerance of salt cress plants compared to Arabidopsis. Arabidopsis (A) and salt cress (B) plants after freezing treatment (−15°C for 24 h). Plants were acclimated for 7 d at 4°C prior to freezing treatment. The photograph was taken 1 week after freezing treatment.

A



B

DNA Content of Salt Cress

	DNA Content (pg/2c nucleus)	
	Arabidopsis	Salt Cress
Average	0.53 ± 0.03	1.00 ± 0.02

Figure 13. A, The chromosome complement of salt cress root cells comprises 14 chromosomes. B, DNA content of salt cress compared to Arabidopsis.

salt cress genes are overrepresented in the small collection of available salt cress ESTs, indicating that they represent abundant transcripts in the RNA from salt-stressed plants (Table II).

Global Analysis of Salt Cress Transcription at High Salinity

High-throughput transcript analyses were conducted in which targets of salt cress were prepared from control plants (in the absence of NaCl) and plants that had been salt stressed (150 mM NaCl) for 3 and 24 h, respectively, for use in Arabidopsis microarray hybridizations (Q. Gong, G. Inan, and S. Ma, ongoing experiments). A preliminary analysis is included here. The cy3- and cy5-labeled targets were hybridized to the full-genome Arabidopsis oligonucleotide microarray platform (http://www.life.uiuc.edu/bohnert/arabarray). Whereas the hybridizations typically produced lower intensities of signals compared to Arabidopsis target hybridizations—due to sequence divergence between the species—the ratio of control to stress target could be used as an indication of either up- or down-regulation of transcripts during a salt stress experiment. Generally, we observed signals significantly above background for salt cress RNA targets for approximately 60% of all Arabidopsis oligonucleotides printed on the arrays. A comparable number for Arabidopsis homologous hybridizations is approximately 80%. Efficient cross-hybridization is documented by examples where an available salt cress EST overlapped with the Arabidopsis oligonucleotide sequence printed (Fig. 14). Compared are sequences, intensity ratios, and actual images for At2g41430 (ERD15) and At1g01720 (ATAF1) and salt cress BM985810 and BM985641, respectively. Also included are control elements for functions that are repeatedly printed in each segment of the microarray slides. The ongoing analyses by microarray hybridization are documented at http://www.life.uiuc.edu/bohnert/thweb, which presents the data for MIAME-compliance. Obviously, a number of stress response-related transcripts in Arabidopsis are expressed at a higher intensity in salt cress even in the absence of stress. It appears that salt stress-induced increases in intensity are significant, but they start from a higher basal level. Table III provides examples in a comparison of microarray and real-time reverse transcription (RT)-PCR data. For nine of ten examples, the fold induction after

Table II. *Salt cress paralogs for Arabidopsis genes*

Accession No.	No. ESTs among Approximately 1,600 Th cDNAs	Th/At Homology (% Amino Acid Identity)	At Gene Number	No. ESTs in At	Function	Remarks/Domains
BQ060238	3	68	At1g28250	4	Unknown protein	At ESTs: flower-stress-mixed; full-length (102 amino acids)
BM985867	2	70	At1g33690	8	Unknown protein	
BM985546	17	49	At1g64370	22	Unknown protein	
BI698758	2	50	At2g16800	6	Unknown protein	NicO high affinity domain
BM985645	4	53	At2g22720	12	Unknown protein	
BM985566	8	42–44	At1g20450	80	Dehydrin-ERD10	
BM985621	2	52	At1g20440	109	Dehydrin-COR47	
BQ079242	4	68	At5g15970	53	KIN2	66 amino acids; full-length, COR 6.6 (kin2)
BQ079196	7	65	At5g15960	7	KIN1	66 amino acids, full-length; 2 ESTs dehydration; 1 EST salinity
BM985779	4	52	At2g18440	18	GUT15	75 amino acids, full-length; unstable transcript group
BM985965	9	50	At1g27540	8	Unknown protein	Contains F-box; ESTs: dehydration cDNA library

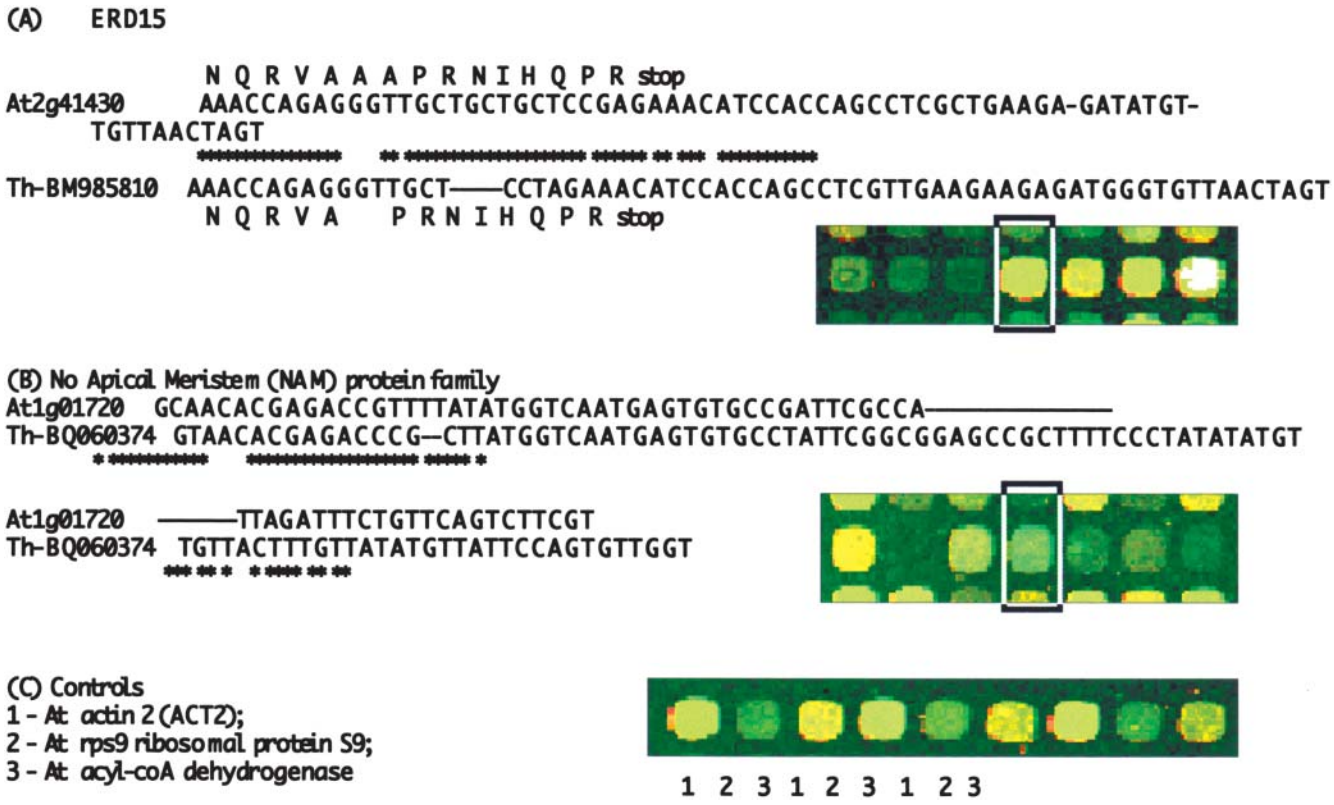


Figure 14. Salt cress gene expression may be recorded using Arabidopsis oligonucleotide-based microarrays. Compared are sequences of two Arabidopsis genes (At2g41430—ERD15; At1g01720—similar to NAC domain protein No Apical Meristem (NAM) GB:AAD17313) with salt cress EST sequences (BM985810, BQ060374). Their signals on the microarray slides are boxed. Also, false-color intensities for three control genes are included. For ERD15, the position of the oligonucleotide printed on microarrays is identified by the single-letter amino acid code, indicating the C terminus of the protein. The comparison with salt cress shows a deletion of two amino acids and several single-nucleotide exchanges in the 3′ UTR.

3 h of salt stress (150 mM) agrees well between the two techniques, with the exception of ABI1. Hybridization of salt cress mRNA to the microarray based on Arabidopsis sequences reports ABI1 (At4g26080) down-regulation. However, when using primers based on a salt cress EST similar to Arabidopsis ABI1 (accession no. BQ079252), up-regulation is observed, suggesting that a paralogous ABI1 exists in salt cress. The putative second ABI1 gene (BQ079252) has itself a homolog among the sequenced Thellungiella ESTs. This sequence (BM985573) is more similar to the authentic Arabidopsis ABI1 sequence (73%) than the putative homolog BQ079252 (65%).

Salt-Sensitive Mutants of Salt Cress

EMS mutagenesis of salt cress seeds followed by screening of T_2 segregating progeny lines for reduced salinity tolerance resulted in the identification of approximately 160 putative mutants with loss of extreme tolerance (*let*). Rescreening of about 50 of these using the root-bending assay (Wu et al., 1996) confirmed the reduced salt-tolerance phenotype of four lines (*let1–let4*). One of these (*let1*) was examined further and its sensitivity phenotype in a root-bending assay was determined (Fig. 15). Salt sensitivity of the *let1* mutant was confirmed in greenhouse experiments using 3-week-old plants (Tables IV and V). When the salt concentration was increased gradually to 300 mM NaCl, shoot growth of *let1* was more strongly inhibited than was the salt cress wild-type control ($n = 20$; $P < 0.001$; Table IV). Likewise, when root elongation of *let1* was evaluated in the presence of either NaCl or KCl, roots of *let1* showed a moderate, but clear, reduction relative to salt cress wild type ($n = 15$; $P = 0.004$; Table V). Segregation of the T_2 seedlings on NaCl medium indicated that the salt-sensitivity phenotype of *let1* is controlled by a single locus (data not shown).

The *let1* mutant accumulates more NaCl during an 18-d exposure than wild-type plants. Mutation at the let1 locus had no apparent effect on Pro accumulation after NaCl exposure (Fig. 16A). It appears, therefore, that the let1 locus is involved in the control of ion accumulation and subsequent effects on growth during NaCl exposure.

DISCUSSION

Salt cress is a small crucifer native to environments characterized by extremely high-salt concentrations.

Table III. *Comparison of expression changes for selected salt cress transcripts*

Compared are selected transcripts for which salt cress clones have been obtained by designing salt cress-specific primers and comparing their salt stress to control (S:C) ratio of expression changes determined by real-time RT-PCR (S:C-PCR) to those determined by microarray hybridization to the Arabidopsis long-oligo microarray platform (S:C-MA). Shown are the $\log_{10}$ ratios for intensity (C*S), and $\log_{10}$ (S:C) ratio from microarray experiments, and the fold-change in microarray (MA) and quantitative real-time RT-PCR (PCR) analysis.

AGI	Function	Length	Log (C*S)	Log (S:C)	S:C MA	S:C PCR
At1g01720	ATAF1	77	3.727	0.632	4.28	4.72
At3g04120	GapC	68	5.353	0.542	3.48	2.43
At1g11910	Aspartic proteinase	94	4.426	0.020	1.05	1.22
At2g41430	ERD15	59	4.634	−0.552	0.28	0.24
At5g66190	Ferredoxin-NADP+ reductase	95	3.642	−0.118	0.76	0.65
At4g26080[a]	ABI1[a]	95	4.115	−0.259	0.55[a]	2.88[a]
At4g11650	Osmotin	70	4.503	−0.438	0.37	0.40
At3g44880	Lls1	94	3.887	0.154	1.43	0.99
At3g20410	CPK9	92	3.801	−0.420	0.38	0.52
At2g38540	LTP1	81	5.341	0.479	3.01	1.41

[a]The microarray signal was obtained by hybridization to the printed Arabidopsis ABI1-specific oligonucleotide; the real-time PCR signal was obtained by using a primer pair for the cloned ABI1 homolog from salt cress (BQ079252). Several salt cress ESTs (gi|19684239; gi|19913611; gi|19855150; BQ07252), annotated as ABI1 homologs, seem to indicate the existence of ABI1 paralogs in salt cress that may be regulated in a different manner.

Further, the salinity stress is also often accompanied by extremes on both sides of the optimum temperature, low humidity, and extreme pH conditions (Rollins, 1993). Clearly salt cress is not only highly tolerant to salinity but also to low-temperature stress, and can thus be considered an extremophilic higher plant. Of the four ecotypes of salt cress that we have collected (one from Colorado, U.S., one from Yukon Territory, Canada, and two from Xinjiang and Shandong Provinces, People's Republic of China), the Shandong ecotype was used exclusively in the experiments reported here. The Shandong ecotype is native to the seacoast of northeast China near the mouth of the Yellow River, but it can be found growing inland on the vast Yellow River flood plain where highly saline soils (2.2% salt or higher) predominate. This region has a temperate climate, and the Shandong ecotype can be classified as a winter annual (after Koornneef et al., 1998) with a strong requirement for vernalization.

Salt Cress Is a Classic Halophyte

Our results clearly demonstrate that salt cress exhibits growth and other properties entirely consistent with those of halophytes. It grows rapidly at moderate salt concentrations and can survive and reproduce at extreme salinity, including near-seawater concentrations by controlled accumulation of high internal levels of NaCl (Fig. 4). Although discussion and debate over the issue of salt accumulation by glycophytes and halophytes has continued for several decades (e.g. Flowers et al., 1977, 1986; Hasegawa et al., 2000), there is little question today that all plant species accumulate Na^+ and Cl^- when exposed to concentrations of these ions much above 20 to 50 mM. The observations by many investigators of the relative rates of net Na^+ and Cl^- ion accumulation and the subsequent consequences on growth, metabolism, and survival between plant species has generated a wide variety of interpretations of the physiological bases of halophytism. It now seems that these differences in interpretations are predominantly a reflection of a single critical attribute that can vary considerably both between and among glycophytes and halophytes. Specifically, the ability to control net Na^+ influx into the cytoplasm and its subsequent sequestration into the vacuole, without accumulation to toxic levels in the symplast, is of overriding importance in determining plant response to salinity (Hasegawa et al., 2000). Great complexity is manifested among halophytes with this trait, however, because this ability is influenced by several effectors, including those that mediate Na^+ influx and efflux across the plasma membrane (Rus et al., 2001; Shi et al., 2002; Tester and Davenport, 2003) as well as vacuolar compartmentalization (Yokoi et al., 2002). These transport systems are all driven by the H^+-electrochemical potential generated by H^+ pumps (Hasegawa et al., 2000). Furthermore, the extent of the signal transduction pathways that control ion homeostasis necessary for salt adaptation is only now being deciphered (Xiong and Zhu, 2002; Zhu, 2002). In addition, these cellular control features can be manifested differently within different cell types with important consequences for overall plant response. For example, the control of Na^+ fluxes at root cortical and xylem parenchyma cells have marked influences on Na^+ movement from root to shoot. Likewise, control of Na^+ fluxes between the xylem and leaf mesophyll cells exerts a powerful effect on ion accumulation in the apoplast of leaf cells. Thus, various combinations of these attributes have led to great variation among species and, subsequently, a considerably complex characterization system for salt tolerance (Greenway and Munns, 1980).

Although some disagreement still exists regarding how glycophytes and halophytes may differ in ion transport to the shoots (Flowers et al., 1977, 1986), an important, generally agreed-upon feature for halophytes in this regard appears to be the minimization of flow of Na^+ ions through the apoplast past the ion-impermeable suberized Casparian strip of the root endodermis (Flowers et al., 1986; Yeo et al., 1987). This so-called bypass flow can significantly influence salinity levels in the shoot system largely via a two-component process. First, Na^+ can bypass the plasma membrane of the endodermis and inner stele parenchyma, sites that are responsible for active (controlled)

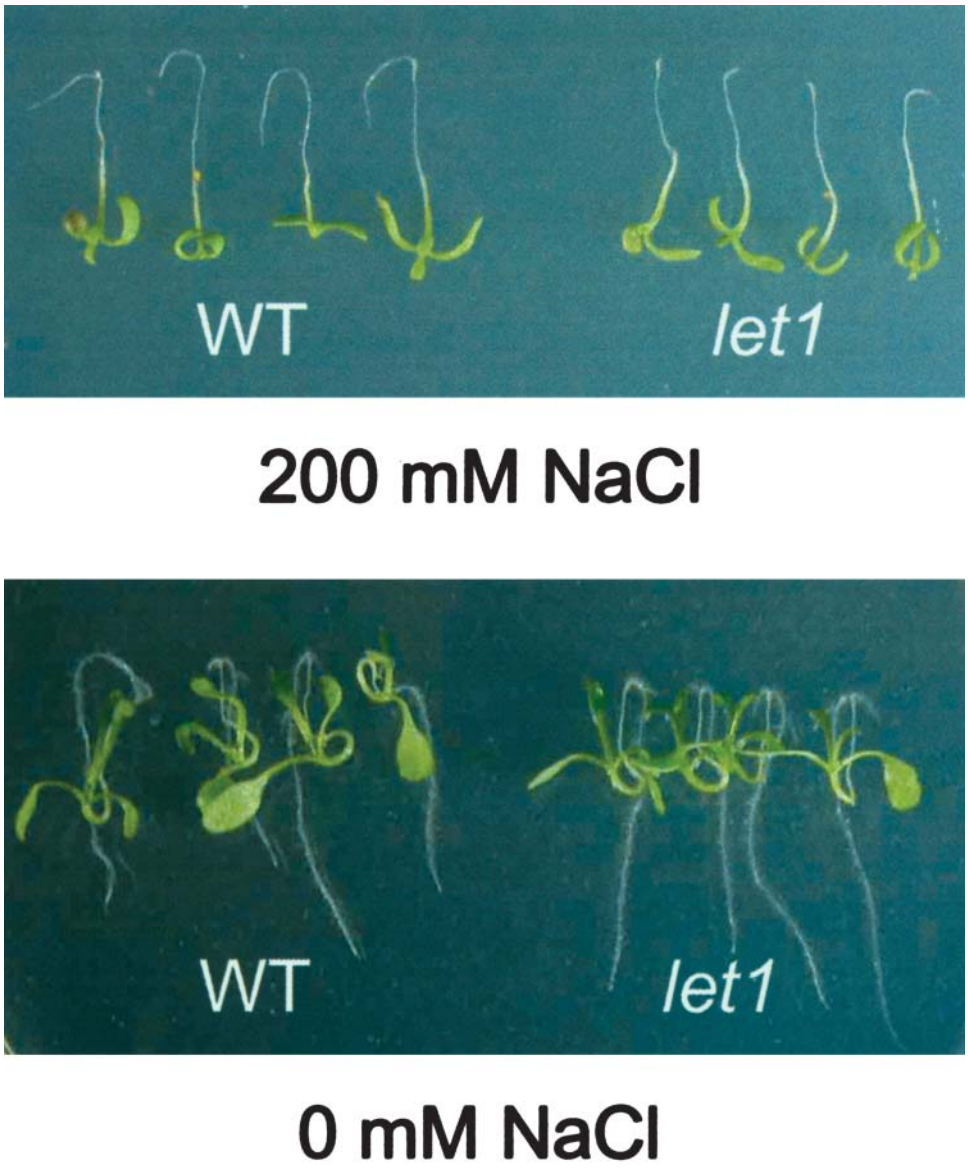


Figure 15. Root-bending assay of wild-type salt cress and the *let-1* mutant. Surface-sterilized seeds were sown on cellophane membrane overlaying an agar surface. After stratification for 3 d at 4°C, plates were incubated at 22°C for 6 d in a vertical position. The membranes with seedlings were then transferred to plates with agar medium containing 0 or 200 mM NaCl and rotated 180°; the photograph was taken 10 d later.

transport of ions into the xylem. High apoplast Na^+ concentrations, slow rates of endodermal differentiation, and disruption of the endodermal barrier during lateral root production each may contribute to greater bypass flow of Na^+ (Yeo et al., 1987; Yadav et al., 1996). Second, movement of ions into the xylem from the apoplast is greatly enhanced by high rates of transpiration (Yeo et al., 1987; Lovelock and Ball, 2002), thereby magnifying the effect of soil salinization on CO_2 uptake, photosynthesis, and overall plant growth and productivity. Thus, minimization of bypass flow in halophytes could improve their performance in high-salinity conditions. Salt cress exhibits features such as a double endodermis, reduced rates of Na^+ accumulation, and osmotic potential decline, as well as reduced transpiration (described below), all of which indicate it is capable of strict reduction of bypass flow (Figs. 4, 7, and 9).

Salt Cress Displays Important Differences from Arabidopsis in Morphology and Stomatal Functions Related to Halophytism

Under both saline and nonsaline conditions, salt cress exhibited a whole-plant transpiration rate much lower than that of Arabidopsis during both day and night periods (Fig. 8), although leaf stomatal densities are higher than observed for Arabidopsis ($n = 9$; $P < 0.001$; Table I). Even though the effect of transpiration on ion transport to shoots could be minimized by reduction or elimination of bypass flow in halophytes with a more effective endodermis, rapid transpiration

Table IV. *The effect NaCl stress on shoot FW of salt cress wild-type and mutant (let1) plants*

Three-week-old plants were grown in soil for 21 d and salinized incrementally, every 5 d, to final concentrations of 0 and 300 mM NaCl. Values are means ± SE, $n = 20$.

	Shoot FW	
NaCl Concentration	Salt Cress Wild Type	Salt Cress *let1*
mM	*g*	
0	3.198 ± 0.151	2.588 ± 0.137
300	0.292 ± 0.015	0.163 ± 0.012[a]

[a]Significantly different from salt cress wild type ($P < 0.001$; Student's *t* test).

would still be expected to increase ion uptake by concentrating ions in the rhizosphere through an increased mass flow of water and dissolved ions toward the roots (Flowers et al., 1986). Therefore, even though high transpiration may reduce the ion concentration in the xylem, it can still increase the net movement of ions to the shoot (Flowers et al., 1986). In fact, the observation that there is not a strict proportionality between the amount of transpiration and the net ion movement to the shoot is often given as evidence that ion loading into the xylem is regulated (Lovelock and Ball, 2002). Thus a double endodermis and more succulent leaves with reduced transpiration rates, together with more controlled ion loading into xylem of salt cress, can reduce the net rate of salt movement to shoot tissues (Fig. 4).

Genes that control morphological/physiological traits that are important to salt accumulation have been discovered in Arabidopsis. For example, genes that affect endodermis development (DiLaurenzio et al., 1996) stomatal density (Gray et al., 2000), and aperture (Wang et al., 2001; Zhu et al., 2002) have been reported previously. Arabidopsis Relative Model Systems (ARMS) such as salt cress offer us, for the first time, the opportunity to examine the role and divergence of such genes in natural evolutionary changes during speciation that control fitness to extreme environments.

Genetic Control of the Regulation of Osmotic Potential, Osmolyte Accumulation, and the Requirement of Osmotic Adjustment for Growth Can Be Addressed with Salt Cress

Mutants of salt cress with disruptions in osmotic regulation will be much easier to isolate than in Arabidopsis because the high-salinity tolerance of salt cress allows osmotic adjustment to affect growth phenotype separately from mutations that affect ion homeostasis. Salt cress plants accumulate large concentrations of Pro in response to salt stress that are sufficient to affect osmotic adjustment (Fig. 5A), but no other compatible osmolyte was detected in significant quantities. The concentrations of Pro measured were sufficient to balance the cytoplasmic osmotic potential with the Na^+ and Cl^- ions that had accumulated, presumably, mainly in the vacuole (Fig. 4). Salt cress does not maintain constitutively high levels of Pro (Fig. 5A), although its leaf osmotic potential was significantly lower than that of Arabidopsis under nonsaline conditions (Fig. 7A). This is likely due to modest accumulation of several other solutes, although we have not yet measured some important, but less common, osmolytes such as sugar alcohols. Even though considerable effort has been made to understand the role of Pro accumulation in stress adaptation (Rhodes et al., 2002), we now have, for the first time, the opportunity to determine the genetic changes controlling Pro metabolism that have evolved to mediate stress tolerance in a halophyte through comparison of gene structure and function between salt cress and Arabidopsis.

In the absence of NaCl exposure, the growth rates of salt cress were similar to those observed in Arabidopsis (Figs. 1A and 2A), even though the osmotic potentials of salt cress were more than 1 MPa lower than those measured in Arabidopsis (Fig. 7A). This indicates that, despite the fact that osmotic potential must be lower than external water potential (turgor pressure must be present) to allow growth, the rate of growth in a specific osmotic environment is not always proportional to cellular osmotic potential. This is clear from the observation that, in the presence of NaCl, Arabidopsis plants were unable to maintain growth even though they showed osmotic adjustment equivalent to that of salt cress for more than approximately 14 d when exposed to external concentrations of 100 mM or higher (Figs. 1, 2, and 7C). Furthermore, leaf turgor pressure in Arabidopsis exceeded 1 MPa when plants were exposed to 100 or 200 mM NaCl (Fig. 7B), and growth was still strongly inhibited relative to

Table V. *The effect of NaCl and KCl stress on root growth of salt cress wild type and EMS mutant (let1)*

Six-day-old seedlings were transferred to agar plates containing MS medium or MS medium supplemented with NaCl or KCl. Root elongation occurring within the 10-d period after transfer is presented. Values are means ± SE; $n = 15$.

	Root Length	
	Salt Cress Wild Type	Salt Cress *let1*
	mm	
NaCl Concentration (mM)		
0	18.20 ± 1.200	16.50 ± 1.10
250	8.63 ± 0.459	6.10 ± 0.77[a]
300	6.99 ± 0.626	4.98 ± 0.69[b]
KCl Concentration (mM)		
0	18.20 ± 1.20	16.50 ± 1.10
250	1.33 ± 0.36	1.12 ± 0.32
300	1.95 ± 0.29	1.29 ± 0.21[a]

[a]Significantly different from salt cress wild type ($P < 0.05$; Student's *t* test). [b]Significantly different from salt cress wild type ($P < 0.01$; Student's *t* test).

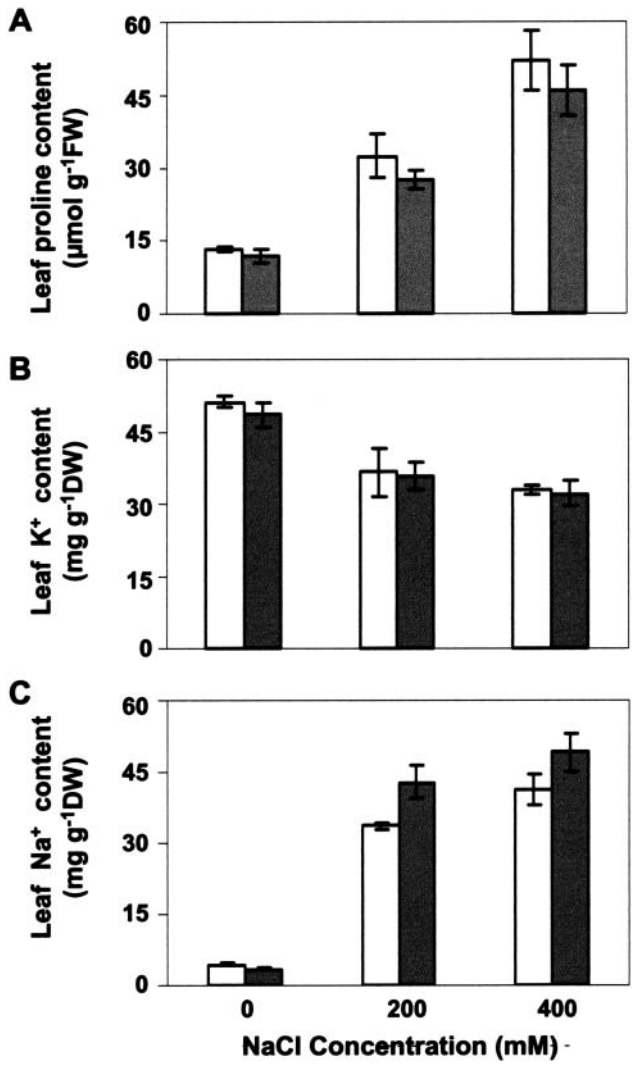


Figure 16. Effect of NaCl on Pro content and ion accumulation in leaves of salt cress wild type and EMS mutant *let1*. Plants were grown in Turface calcined clay and irrigated with 0, 200, and 400 mM NaCl for 18 d. White bars, salt cress wild type; black bars, salt cress mutant *let1*. Levels of Pro and Na^+ and K^+ were determined as described in "Materials and Methods." Values are means ± SE; $n = 3$. Leaf Na^+ content of salt-treated salt cress wild type and EMS mutant *let1* are significantly different from each other ($P < 0.05$), while leaf K^+ content and leaf Pro content are not significantly different at $P = 0.05$; Student's *t* test analysis was done by using grand means.

salt cress. A similar response was reported by Bressan et al. (1990), who demonstrated that osmotic adjustment and concomitant high turgor levels were unable to maintain growth in tobacco cells adjusted to high levels of external NaCl. Thus, even though osmotic adjustment and turgor maintenance are necessary for growth, they are not sufficient. It seems likely that some other regulated processes besides osmotic adjustment must allow salt cress to maintain high growth rates when exposed to high external NaCl. Such processes may involve ABA-mediated growth control. For example, a recently described Arabidopsis ABA biosynthesis mutant, *sto1/nced3*, that fails to increase ABA levels and osmotically adjust compared to wild-type plants, also grows faster in the presence of high external NaCl (B. Ruggeiro, unpublished data).

Molecular Genetic Features of Salt Cress Allow Efficient Identification of Mutants

The strong vernalization requirement and a tendency for nonsynchronous germination may reduce the speed and efficiency of genetic analysis of salt cress compared to Arabidopsis, but salt cress possesses several attributes of a superb genetic model that more than compensate for these moderately unfavorable features. The advantages of short life cycle, small size, and prolific seed production of salt cress have already allowed us to obtain EMS mutants of the plant, including lines that require no vernalization (J.-K. Zhu, unpublished data), mutants that germinate on 300 mM NaCl (R.A. Bressan, unpublished data), and several lines showing a significant loss of salt tolerance, including *let1* (Table IV).

Although we have not identified the gene underlying the salt-sensitive phenotype of *let1*, the genetic segregation of salt sensitivity of *let1* and the other three stable mutants confirms the existence of individual genetic loci in salt cress that are critical to NaCl tolerance. Two possible routes to the identification of mutant loci in salt cress exist. In the case of EMS-induced mutations, loci could be located using a mapping approach based on DNA polymorphisms. This will require the existence of ecotypes of salt cress that would allow the development of a DNA marker system for positional cloning. We have identified three additional ecotypes of salt cress from geographically divergent native habitats that should offer a possibility for the detection of DNA polymorphisms. Also, because of the close relationship between salt cress and Arabidopsis, it may be possible to utilize markers from Arabidopsis in mapping. All of the salt cress ecotypes show similar levels of salinity tolerance and, in preliminary studies, appear to be cross-fertile with our original ecotype from Shandong (Z. Cao, unpublished data). The high degree of gene sequence identity with Arabidopsis indicates that isolation of Arabidopsis orthologs from salt cress should be efficient and that design of antisense or double-stranded RNA constructs for RNAi gene silencing should be possible. It is also likely that promoter switching will be a feasible approach to test the importance of halophyte promoter function in salt tolerance. The ease of transformation of salt cress by the floral dip procedure should greatly facilitate implementation of such strategies.

The Use of ARMS Will Facilitate Understanding Evolutionary Adaptation to Extreme Environments

Even though the comparison of EST sequences from Arabidopsis and salt cress revealed a high DNA sequence identity (90%–95%) for the majority of transcripts (http://www.life.uiuc.edu/bohnert//

projects/thel.html), it seems significant that sequence identities for a number of genes that are known to function in abiotic stress tolerance in Arabidopsis displayed much lower identity scores (Table II). The significant deviation in sequence identity and a detailed analysis of the encoded reading frames indicated that many of the low-identity sequences appear to be paralogs of genes found in Arabidopsis. It is possible that salt cress includes a larger number of paralogs for stress-related functions compared to Arabidopsis. Such an outcome, if demonstrated, would carry significant implications for evolutionary adaptive theory. This possibility may also explain a significantly larger number of abundant transcripts in salt cress-encoding enzymes involved in oxygen radical scavenging that are aligned with a single Arabidopsis isolog, whereas probability scores are widely divergent. We hypothesize that the stress-relevant transcriptome of salt cress has undergone significant adaptive changes in gene complement and, subsequently, in sequence after reproductive separation from the clade in which Arabidopsis is located, and that this evolutionary adaptation generated the observed fitness to extreme environments. A further example of this can be seen from the *SOS1* gene that we know is required for salt tolerance in Arabidopsis (Zhu, 2001b). The *SOS1* gene from salt cress displays only 84% sequence identity with *SOS1* of Arabidopsis, again exemplifying greater divergence than average for the 1,600 randomly selected ESTs of salt cress.

Transcriptome Analysis of ARMS May Utilize Arabidopsis Microarray Tools

Microarray analyses are ongoing with RNA from plants that have experienced different stress regimes. As yet, there is no clear understanding about the level of salinity that might constitute a comparable stress in Arabidopsis and Thellungiella. However, some important novel conclusions seem to emerge. Components of the salinity stress defense and survival machinery seem to be expressed at a higher level in salt cress even in the absence of stress, compared to Arabidopsis (http://www.life.uiuc.edu/bohnert/thweb). Also, the amount of transcripts of relevant transcription factors and signal transduction components are less dramatically, yet significantly, up-regulated during salt stress in this halophyte, reflecting perhaps a lower necessity for transcription regulation under stress. This observation is supported by real-time quantitative RT-PCR analyses for 10 regulated transcripts that have been selected at random (Table III). Also, both the EST expression profile, albeit based on a small number of sequences, and the comparative real-time RT-PCR and microarray data point toward a fundamental difference between Arabidopsis and salt cress with respect to the evolutionary emergence of paralogous genes in salt cress that enhance abiotic stress response pathways.

Salt Cress Fills the Need for a Halophyte/Cryophyte ARMS

Our knowledge of the genetic basis of salt and cold tolerance in Arabidopsis (Hasegawa et al., 2000; Thomashow, 2001; Zhu, 2001a, 2001b, 2002; Zhu et al., 2002; Sung et al., 2003) now enables us to pose explicit hypotheses regarding the special attributes and mechanisms that permit high tolerance and productivity of plants that are naturally tolerant to extreme salinity and cold, such as: (1) high tolerance is due to special promoter structures; (2) extremophytes have more activated forms of genes or gene products that function in tolerance; or (3) extreme tolerance results from unique genes. The resolution of these hypotheses (Schroeder et al., 2001; Zhu, 2001b; Xiong et al., 2002) is crucial to agriculture and food security where extreme environments severely limit plant growth and survival. However, these hypotheses cannot be tested with Arabidopsis alone. We will not be able to determine the genetic bases of those specialized mechanisms without effective extremophyte genetic models. Results presented here demonstrate that salt cress meets all the criteria of an effective molecular genetic model system and has characteristic mechanisms of salt tolerance common to halophytes.

Salt Cress and Other Relatives of Arabidopsis Will Be Useful for the Study of Adaptation to Many Types of Extreme Environments

Besides salt and cold tolerance, salt cress may possess undiscovered tolerances. Another Arabidopsis relative with model characteristics displays extreme tolerance to heavy metal toxicity (Persans et al., 2001). The perennial Arabidopsis relatives *Descurainia pinnata* and *T. parvula* possess genetic model features and extreme desiccation and flooding tolerance, respectively (R.A. Bressan, unpublished data). The results presented here clearly open the possibility to identify and use many other ARMS. ARMS will extend our use of the formidable tools developed for Arabidopsis to understand how plant speciation has resulted from genetic divergence for fitness to a wide variety of environments, as well as to identify genes that function in other traits of agricultural importance such as heat tolerance, nutrient deprivation, perennialism, and specific tolerances to diseases and pests.

MATERIALS AND METHODS

Plant Materials and NaCl Treatments

Arabidopsis ecotype Columbia and salt cress (*Thellungiella halophila*; ecotype Shandong wild-type and EMS-mutagenized mutant ([*let1*]) were grown in pot media (Metro Mix 360, Scotts-Sierra, Marysville, OH) in a greenhouse under 21°C-day/8°C-night temperature and 16-h photoperiods. One week prior to treatments, seedlings were transferred to Turface calcined clay (Profile Products, Buffalo Grove, IL) in 7.5-cm pots. These were placed in a growth chamber that provided a photosynthetic photon flux of 250 μM m^{-2} s^{-1} from cool-white fluorescent bulbs in 16-h photoperiods. Day and night temperatures were set at 22°C and 19°C, respectively. Plants were irrigated with a nutrient solution containing 200 mg N L^{-1} supplied from 1,000 mg L^{-1}

15-5-15 commercial fertilizer formulation (Miracle Gro Excel Cal-Mag; Scotts-Sierra) every other day, and treatments were applied by the addition of NaCl at the desired concentrations in fertilizer solution. The NaCl treatments were applied either by direct application of the desired concentration or by incremental increases of NaCl in the irrigation water until the final desired concentrations were reached.

Seeds used in germination experiments were surface sterilized briefly in a solution of 70% ethanol, followed by 20% (v/v) commercial bleach for 10 min. They were then washed with sterilized water four times and suspended in sterile 0.3% (w/v) low-melting agarose before sowing on agar Murashige and Skoog (MS) plates (Murashige and Skoog, 1962). Plates were stored at 4°C for 48 h to synchronize germination and then incubated at 22°C under continuous illumination.

Growth Measurements

Leaf and root FWs were determined immediately after harvesting, and samples were dried in an oven at 65°C for 2 d to obtain DWs. For root length measurements, 6-d-old salt cress wild-type and mutant (*let1*) seedlings were transferred to MS agar plates supplemented with various NaCl concentrations, and their roots were arranged pointing downward on vertically positioned plates. Root length was marked at the onset of treatment, and the increase in length was measured after 10 d.

Leaf Water Relations

Leaf water potential was measured on single leaves by use of a Scholander-type pressure chamber (PMS Instruments, Corvallis, OR). Leaf samples were then frozen in sealed polyethylene freezer bags, thawed, and centrifuged at 1,000*g* for 20 min at 6°C to 8°C to extract cell sap. Osmotic potential of cell sap was measured by using a Wescor Model 5100C vapor pressure osmometer (Wescor, Logan, UT). Leaf turgor pressure was estimated as the difference between water potential and osmotic potential, and data were combined over all sample dates. Stomatal conductance was measured with a CIRAS-1 portable photosynthesis system (PP Systems, Amesbury, MA). Whole-plant water loss rates were determined throughout diurnal time courses by a gravimetric procedure. Pots were sealed in plastic wrap to prevent soil evaporation and were weighed every 30 min for 7 d. Succulence was calculated as the ratio of shoot FW to shoot DW.

Na^+ and K^+ Ion Content Determination

The harvested seedlings of treated and control plants were rinsed with deionized water and dried at 65°C for 2 d. One hundred milligrams of dry leaf material were then extracted with 10 mL of 0.1 N HNO_3 for 30 min and then filtered through Whatman number 1 filter paper. Na^+ and K^+ contents in the solutions were determined by using an atomic absorption spectrophotometer (Varian, Victoria, Australia; SpectrAA-10).

Pro Content Determination

A sample of 0.2 g of freshly harvested leaves was extracted in 10 mL methanol overnight at 4°C, phase separated by the addition of 2.5 mL chloroform and 2.5 mL water, and then stored at 4°C for 2 h. The upper aqueous layer was removed and transferred to new vials and concentrated to dryness under a stream of N_2 at 30°C. Samples were redissolved in 2 mL water. Free Pro content was measured in 0.1-mL samples, according to the method described by Bates et al. (1973). Pro levels were confirmed and other free amino acids were measured by gas chromatography, according to Rhodes et al. (1986).

Total Soluble Sugars

A sample of 0.5 g of harvested leaves was crushed in 5 mL of 95% (v/v) ethanol. The insoluble fraction was washed twice with 5 mL of 70% (v/v) ethanol, and all soluble fractions were centrifuged at 3,500*g* for 10 min. The supernatants were collected and stored at 4°C. Total soluble sugars were analyzed by reacting 0.1 mL alcoholic extract with 3 mL freshly prepared anthrone and 100 mL of 72% (v/v) H_2SO_4, followed by immersion in boiling water for 10 min. After cooling, the A_{625} was determined in a spectrophotometer.

Stomatal Density, Epidermal Pavement Cell Density, and Stomatal Index

Stomatal density, epidermal pavement cell density, and stomatal index for adaxial surfaces of leaves were determined using bright-field light microscopy modified from Gray et al. (2000). Leaf surface imprints were created using cyanoacrylate adhesive (Duro Super Glue; Manco, Avon, OH). Leaves selected for sampling were of uniform size and age. For Arabidopsis, these were taken from the 24th to 26th leaf from the first leaf, while for salt cress, rosette leaves were selected for uniform size and cauline leaves were selected from the third node. Leaf samples from nine to twelve plants were taken from the middle of the blade between the midrib and leaf margin. Arabidopsis plants were 39 d old at the time of sampling. In salt cress, rosette leaves were sampled prior to bolting, while cauline leaves were sampled after bolting (7 weeks and 9 weeks old, respectively). Stomatal index was calculated as: (stomatal density × 100)/(stomatal density + pavement cell density).

Cold Tolerance

Plants of Arabidopsis ecotype Columbia and salt cress ecotype Shandong were grown in soil in a growth chamber (23 ± 2°C, 16-h-light and 8-h-dark cycles) for 4 weeks and then incubated at 4°C under white fluorescent light (16-h light and 8-h dark cycles) for 1 week for cold acclimation. After cold acclimation, the plants were subjected to freezing temperature (−15°C) for 24 h. The plants were then transferred immediately to 4°C under white fluorescent light for 24 h. Seedling damage was scored 7 d later after removal to the greenhouse.

cDNA Libraries, EST Sequences, and Microarray Analyses

Salt cress cDNA libraries were generated in λ-ZAP Express (Stratagene, La Jolla, CA) and phagemids were excised and cloned into the vector pBK-CMV. The libraries were made from plants that had been stressed by the addition of 250 mM NaCl for 24 h (http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?CMD=search&DB=nucleotide; "Thellungiella"). Sequences deposited into EST database were retrieved, annotated, in comparison to The Arabidopsis Information Resource (TAIR) and The Institute for Genomic Research (TIGR) Arabidopsis databases, and analyzed by FASTA analysis.

To avoid possible effects of diurnal or circadian rhythms, tissues from treatment and control plants were harvested at identical time points. To account for differences among plants, tissues from more than 10 plants were combined prior to RNA extraction. Leaf tissues were finely ground in liquid nitrogen using a mortar and pestle. Total RNA was extracted from 3 to 5 g of ground tissue using 10 mL of the TRIzol reagent (Gibco/BRL Life Technologies, Invitrogen, Carlsbad, CA). mRNA isolation was performed using the Poly(A) Track kit (Promega, Madison, WI). Target production for microarray hybridization was performed by incorporation of fluorescent nucleotide analogs during first-strand reverse transcription using mRNA templates by established methods. Targets were vacuum dried in the dark and dissolved in hybridization buffer containing 25% (v/v) formamide, 5× SSC, 0.1% (w/v) SDS, and 10 μg mL^{-1} salmon sperm DNA. Targets were hybridized to a 26,000-element (70-mer oligonucleotides, synthesized by Qiagen, Valencia, CA; printed at the University of Arizona) microarray slide covering the Arabidopsis-transcribed genome (Galbraith, 2003).

To avoid bias in the microarray evaluation as a consequence of dye-related differences in labeling efficiency and/or differences in recording fluorescence signals, dye labeling for each paired sample (stressed/control) was reversed in two individual hybridizations. Three independent hybridizations were performed for pairs of biological samples. The targets were denatured for 2 to 3 min at 95°C and 75 μL was applied to the slides, which were then covered with a coverslip and hybridized for 24 to 48 h at 42°C in high humidity. Slides were washed in 1× SSC, 0.2% (w/v) SDS (4 min at 42°C), 0.1× SSC, 0.2% (w/v) SDS (4 min), and 0.1× SSC (4 min) and dried by centrifugation.

The signal intensity for each array element was captured by scanning using a ScanArray 3000 (GSI-Lumonics, Billerica, MA). The images were analyzed using ImaGene 4.1 software (BioDiscovery, Los Angeles). The scanned data were normalized globally, assuming that the values of $\log_2$ (treatment/control) for most genes should be close to zero (Deyholos and Galbraith, 2001). The cutoff score for up- or down-regulation was defined using criteria that were applied to the mean values of a complete data set produced by dye-reversal hybridizations; if the $\log_{10}$ ratio of salt stress to control for a particular

array element was ≥ ± 0.4 (± 3-fold) and its Coefficients of Variation was less than approximately 40%, the element was classified as significantly different.

Quantitative Real-Time RT-PCR

Quantitative real-time RT-PCR experiments were conducted to verify microarray hybridization results. Three micrograms of RNA from salt cress 3-h control and salt-stressed (150 mM NaCl) materials were used for first-strand cDNA synthesis under conditions identical to those used in the hybridizations. The following primers were used:

At1g01720cF/R (F: 5′-GATTCGGTGCCGAAGCTG-3′; R: 5′-ACCTCGCTCGTGAACTCC-3′); At3g04120cF/R (F: 5′-GCACCACTAACTGCCTTGCT-3′; R: 5′-CATAAGACCCTCAACAATTCCA-3′); At1g11910cF/R (F: 5′-TCTAACCTCTGGGTGCCATC-3′; R: 5′-TCTCATATGTGCTTGAACGTGA-3′); At2g41430cF/R (F: 5′-GAAAAGCCAGCGAAATGG-3′; R: 5′-CGAGGCTGGTGGATGTTT-3′); At5g66190cF/R (F: 5′-TCCATACCCTTAAGACCACACA-3′; R: 5′-CATTCAGACAAGAATGGCAGAG-3′); At4g26080cF/R (F: 5′-GCGATTCAAGGGTTTCGTTA-3′; R: 5′-GGAATTGATCCGAGAGGACA-3′); Th-gi|19684239-cF/R (F: 5′-GCGATTCAAGGGTTTCGTTA-3′; R: 5′-GGAATTGATCCGAGAGGACA-3′); At4g11650cF/R (F: 5′-ATTGCACTGGTGGACTTCAAT-3′; R: 5′-TTCAAAGCGTACTCAGCCAAC-3′); At3g44880cF/R (F: 5′-AATTCGTTGCTCCTTGCTATTC-3′; R: 5′-GAGCAAATCCAAATAACCCATT-3′); At3g20410cF/R (F: 5′-CAGTTCGGGGTCACGTATCT-3′; R: 5′-GCTTTCGTCACCAGCTTCTT-3′); At2g38540cF/R (F: 5′-GAAGTTGGCATGCTTGGTCT-3′; R: 5′-ACGGTTCCACAGCTAAGAGC-3′).

Salt cress actin cDNA primers were used as the internal control in real-time RT-PCR analyses. Detection of RT-PCR products was done by incorporation of the fluorescent dye SYBR green using the QuantiTect SYBR Green PCR kit (Qiagen) following the manufacturer's recommendations. Real-time quantitative determination used the Cepheid Smart Cycler (Cepheid, Sunnyvale, CA), with 5-fold diluted (sterile ddwater) first-strand cDNA reaction mixes. All reactions were heated to 95°C for 15 min, followed by 40 cycles at 94°C for 15 s, at 60°C for 30 s, and 72°C for 30 s.

Light Microscopy and Other Methods

Leaves and roots of Arabidopsis and salt cress plants were sectioned and prepared for light microscopic observations using an Olympus microscope (Olympus, Lake Success, NY). Tissues were fixed in 3% (w/v) glutaraldehyde and 2% (w/v) formaldehyde in 0.05 M phosphate buffer (PB), pH 6.8, post fixed in 2% (w/v) osmium tetroxide in PB, washed with PB, and dehydrated in an ethanol series. Samples were embedded in LR White resin (hard grade; Ted Pella, Redding, CA). Then 0.1-μm sections were prepared on a Reichtert-Jung 1140 rotary microtome and stained in 0.05% (w/v) toluidine blue. The chromosome number of salt cress root cells was determined by the drop-spreading technique, following the procedure of Andras et al. (1999). The DNA content of the salt cress genome was determined by flow cytometry, according to Galbraith (1990).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers BM985810 and BQ060374.

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