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Physiological and molecular strategies for salt tolerance in *Thellungiella halophila*, a close relative of *Arabidopsis thaliana*

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Thesis submitted for the degree of doctor of philosophy

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Declaration

All the work presented in this thesis is my own including the collection and analysis of all the data and the writing up of the thesis. Experimental work for the thesis was carried out at University of Glasgow with the exception of the ^{22}Na flux experiments which were performed at University of Cambridge (under the supervision of Dr. Romola Davenport, Department of Plant Sciences).

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Abbreviations

ABA	Abscisic acid
AFGC	Arabidopsis Functional Genomics Consortium
AMT array	Arabidopsis membrane transporter microarray
ANOVA	Analysis of variance
BSA	Bovine serum albumin
CAM	Crassulacean acid metabolism
CNGC	Cyclic-nucleotide gated channel
DAG	1,2-sn-diacylglycerol
ddH_2O	Double distilled water
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
E_K	The equilibrium potential for potassium
EST	Expressed sequence tag
FDR	False discovery rate
FW	Fresh weight
ICP-OES	Inductively coupled plasma optical emission spectroscopy
iGA	The iterative group analysis
IP_3	Inositol 1,4,5-triphosphate
JA	Jasmonic acid
KOR	K outward rectifier
KIR	K inward rectifier
MES	2-(N-Morpholino)ethanesulfonic Acid
MNS	Minimum nutrient solution
NSCC	Non-selective cation channel
OD	Optical density
P5CS	Delta1-pyrroline-5-carboxylate
PA	Phosphatidic acid
PCR	Polymerase Chain Reaction
RAFL array	The RIKEN Arabidopsis full-length cDNA microarray
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RP	Rank product
RT-PCR	Reverse transcriptional polymerase chain reaction
SA	Salicylic acid
SDS	Sodium dodecyl sulfate
SEM	The standard error of the mean
SSC	Saline-Sodium Citrate
T-DNA	Transfer DNA
TEA	Tetraethylammonium
TMS	Transmembrane spanning domain
VIC	Voltage-independent channel.

Terminology

- 'Concentration':Ion concentrations are given in mM (M, μM, nM etc.). Tissue ion concentrations are given in mg ion per gram FW or DW plant material as indicated.
- 'Up/down regulation'/'transcriptional regulation': these terms should be read in the following context: microarrays measure levels of transcript; the amount of transcript can be changed by regulation at the transcriptional level but also by a change in mRNA turnover.
- Ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, H^+, Cl^-, Cs^+, Rb^+, TEA^+, La^{3+}, Gd^{2+}) are generally written without their charge.
- 'Arabidopsis', 'Thellungiella' in this thesis refer to *Arabidopsis thaliana* ecotype Columbia 0 and *Thellungiella halophila* ecotype Shandong respectively.

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Abstract

Salt stress is one of the most threatening environmental stresses reducing the global food production. Understanding mechanisms of salt tolerance in halophytic plants is a requirement for developing crop species with increased salt tolerance. This study focused on investigating ion transport features in a halophytic relative of Arabidopsis, both at physiological and transcriptional level.

A comparative approach was adopted in this study using the glycophytic model plant *Arabidopsis thaliana*, and its halophytic close relative, *Thellungiella halophila*. Net ion uptake and unidirectional Na fluxes during salt stress were analyzed in the two species. Furthermore, transcriptional profiles of ion transporters under control and high-salt conditions were compared between the two species.

The considerable amount of data produced in this study provide important information for future physiological and molecular studies of both Arabidopsis and Thellungiella. The main results can be summarized thus:

1. After salt stress Thellungiella accumulates less Na in the shoots than Arabidopsis. Net uptake of Na into both roots and shoots was slower in Thellungiella than in Arabidopsis.
2. Lower unidirectional Na influx into root cells is the main reason for the lower Na accumulation in Thellungiella than in Arabidopsis.
3. Voltage-independent cation channels (VICs) are likely to be the Na uptake pathway in both Thellungiella and Arabidopsis.
4. Microarray analysis showed that after salt stress both species showed a tendency to reduce Na uptake by decreasing the expression of possible pathways for Na influx. However, transcriptional control of putative Na transporters ocurred in Arabidopsis in the shoots, whereas it ocurred in Thellungiella in the roots.
5. CNGC8 is a likely candidate for a Na uptake pathway in both Arabidopsis and Thellungiella. Transcript levels of CNGC8 decreased during salt stress in Thellungiella roots and Arabidopsis shoots.

Chapter 1 Introduction

This chapter begins by stressing the importance of research into plant salt tolerance. After a brief outline of salinity induced damage to both agricultural yield and growth of individual plants, strategies that plants adopt to deal with salinity are discussed, and current biotechnological efforts towards producing salt tolerant crops are summarised. The second half of this chapter discusses the experimental design of the thesis, including a description of the model systems, the research techniques and the questions addressed in this study.

1.1 Global food production and the salinity problem

The fast growing world population puts a constant pressure on global food production. Since the 1960s the combined applications of improved water control, intense chemical input, selected crop varieties and engineering of transgenic crops have significantly increased global food production (Huang et al., 2002). In Asia improved crop yield from the 'Green Revolution' reduced the proportion of the population suffering from chronic hunger from 40% to 20% in the 1960s (Toenniessen et al., 2003). However over the same period of time the overall population at least doubled (Toenniessen et al., 2003). Expectations are high for continued progress in traditional breeding practices, biotechnology research and agronomic techniques to further improve food production. During the five years since 1991, public agricultural research expenditure has expanded steadily, especially in developing countries at 3.6% per year (Pardey and Beintema, 2001). Meanwhile the world investment in agricultural research reached US$33 billion per year (Pardey and Beintema, 2001).

Salinity is one of the major abiotic stresses limiting world food production. Irrigation increases yields of most crops by 100 to 400%. By 1999, 42% of arable land in Asia was irrigated. However excessive irrigation causes not only waterlogging in the field

but also soil salinization. After evaporation, concentrated salts precipitate and remain in the soil. Inefficient agricultural practices speed up soil salinization worldwide. According to an FAO estimation in 2002, about 1/10 of the world irrigated land was damaged by soil salinization, and this figure is increasing by 1 to 2% every year. Considering that about 40% of the world food production comes from 17% of the world irrigated land, soil salinization is a dangerous threat to global food production (FAO, 2002), Research in water control was intensively funded, e.g. in India (Fan et al., 1999)and China (Fan et al., 2002), to prevent further soil salinization. Alternatively, developing salt-tolerant crops will expand the territory of global arable land, by making productive use of dry-land and salt damaged land.

1.2 Effects of salinity on plant growth and development

Most plant species including most crops are sensitive to salt (NaCl). Moderate salinity reduces the growth of glycophytic species and increases their susceptibility to other stresses such as drought stress and pathogen attacks (Thaler and Bostock, 2004). Severe salinity affects not only crop yield (Almodares and Sharif, 2005; Murkute et al., 2005; Navarro et al., 2005; Saqib et al., 2004) but also product quality of crops. For example, juice content, titratable acidity and total soluble solid content of meolon were altered when irregated with saline water (Garcia-Sanchez et al., 2003).

Salinity affects seed germination of glycophytic species, including wheat (Al-Ansari, 2003), maize (Ashraf and Rauf, 2001), rice (Asch and Wopereis, 2001) and barley (Huang and Redmann, 1995). Germination rate decreases with increasing NaCl concentrations, while germination time increases with salt concentration (e.g. *Medicago sativa* (Esechie, 1993) and *Sorghum bicolor* (Esechie, 1994)). Priming with calcium solution before germination increases germination rate under saline conditions (Ashraf and Rauf, 2001).

Salinity reduces plant growth. A concept of two-phase growth response to salinity was introduced by (Munns, 1993). The first phase of growth reduction due to hyper-osmotic shock is quickly apparent with little genotypic variation, and usually lasts from days to weeks depending on species. Growth of leaves is more affected than growth of roots in this stage. Symptoms of salt injury show in older leaves. The second phase of growth reduction develops after excessive salt accumulates in transpiring leaves to a toxic concentration that limits growth of the younger leaves by reduced supply of carbohydrates to the growing cells. Growth of cultivars with different salt sensitivities differ in this stage. The two-phase growth response has been demonstrated with maize (Cramer et al., 1994; Fortmeier and Schubert, 1995), wheat (Munns et al., 1995) and rice (Yeo et al., 1991) cultivars.

Salinity has a negative impact on plant development and seed production. Flowering and maturity of rice is delayed by salt stress during both vegetative and reproductive growth stages (Castillo et al., 2004). Even less than 4 g/l NaCl delayed flowering of iris plants by up to 3 days, and the delay of flowering continued after the salt stress was withdrawn (Van Zandt and Mopper, 2002). Sodium accumulation in pollen and stigma reduces pollen viability and stigma receptivity in salt stressed rice lines (Khatun et al., 1995).

1.3 The physiology of salt stress in plants

High concentrations of salt in soil solution decrease the water potential surrounding root epidermal cells. Plant will lose water instantly and lose turgor. Hormonal signals from roots control plant growth (Munns, 2002). In addition to disruptions of balance in water potential under high salt stress, plants suffer from the toxic effect of sodium (Na) and cloride (Cl). In several plant species (e.g. citrus (Fernandez-Ballester et al., 2003; Moya et al., 2003)) salt sensitivity is linked to high Cl rather than high Na concentration in the

soil. The physiological reasons for Cl toxicity in plants are yet to be studied in detail. Even less is known about the transporters involved in the allocation of Cl in different tissues and cellular compartments during salt stress. Because plant cells have a very negative membrane potential uptake of Cl even if present at fairly high concentrations in the external medium has to be coupled with cation uptake (usually protons; (Scheel et al., 2005). By contrast Na is taken up by the root epidermis cells down the electro-chemical gradient at no energy cost. Na accumulation is correlated with salt sensitivity in many crop species (e.g. wheat; (Munns and James, 2003). For most plants Na is not an essential nutrient, rather toxic because of its adverse effects on nutrition, cytosolic enzyme activities, photosynthesis, and metabolism (Shi et al., 2000; Zhu, 2001b). At low levels of salinity, Na compartmentalized in the vacuole can act as an osmoticum to help restore turgor without increasing the cytosolic Na concentration. In this function low mM concentrations of Na can be beneficial for their growth, for example, under conditions of low external K concentrations (Maathuis and Amtmann, 1999). However when salt accumulation exceeds the storage capacity of vacuoles, Na will build up rapidly in the cytoplasm. Na ion has similar physico-chemical features as K, an important macronutrient for plant growth, and therefore competes with K for binding sites on enzymes in the cytoplasm, thus disrupting normal metabolic activities (Murguia et al., 1995). In general, the salt stress response of plants consists of ion homeostasis, osmotic adjustment, detoxification (*i.e.*, damage control and repair) and growth regulation (Zhu, 2002).

1.3.1 Ion Homeostasis

From an energetic viewpoint, it is preferable to control ion uptake initially rather than spending energy on detoxification and damage repair. *In vitro* experiments showed no evidence for higher anti-oxidative resistance in the enzymes of halophytic species

compared to glycophytes (Flowers et al., 1977; Greenway and Munns, 1980). Therefore cytoplasmic Na concentration must be regulated by the plants tolerant to salt stress.

Plant cells have mechanisms to buffer excess ions temporarily because of the presence of large, membrane-bound vacuoles (Apse et al., 1999; Glenn et al., 1999). Three mechanisms may function cooperatively to prevent the accumulation of Na in the cytoplasm: restriction of Na influx, active Na efflux, and compartmentalization of Na in the vacuole (Niu et al., 1995; Shi et al., 2000). It is likely that most plants combine all three strategies. However depending on environmental conditions, plant species and tissue type, the relative proportions of the three factors will vary (Flowers et al., 1977; Greenway and Munns, 1980).

1.3.1.1 Pathways for Na transport

The pathway of Na uptake into plant roots has not been fully identified. Na was considered to be taken up through potassium (K) channels based on the similarity between Na and K ions. Potassium outward rectifiers (KORs) are usually less selective amongst cations than potassium inward rectifiers (KIRs) (K/Na around 10 compared to K/Na around 50 respectively; (Maathuis and Amtmann, 1999). Since under high-salt conditions the driving force for Na is directed inward even at voltages more positive than the equilibrium potential of K (E_K) Na uptake through KORs was considered possible (Schachtman et al., 1991). Outward-rectifying channels that do not distinguish between K and Na have been identified in the xylem parenchyma of barley (Wegner and De Boer, 1997). These channels could provide a pathway for Na re-absorption from the xylem. However, a role of KORs in Na uptake has so far not been demonstrated and they are unlikely to make a major contribution to Na uptake (Amtmann and Sanders, 1999).

HKT1 is a homologue to TRK-type high-affinity K transporters of yeast and bacteria. Depending on the plant species and the individual isoform HKT acts as a K/Na co-transporter or a Na uniporter (Horie et al., 2001; Rubio et al., 1995; Uozumi et al., 2000). A mutation in *AtHKT1* suppressed both the salt-sensitive and the K-deficient phenotype of *sos3* mutants (Rus et al., 2001). SOS3 is a cytoplasmic Ca-binding protein that regulates the plasma membrane Na/H antiporter SOS1 via a protein kinase SOS2 (SOS signalling pathway). The experiments by Rus and collegues (2001) demonstrated that AtHKT1 is not a high-affinity K transporter, but plays an important role in Na uptake under high-salt conditions. Recently Berthomieu *et al.* (2003) reported that another *AtHKT* mutation, *sas2,* displayed increased Na sensitivity, which was accompanied by over-accumulation of Na in the shoots and under-accumulation in the roots, compared to wild-type plants (Berthomieu et al., 2003). Localization of *AtHKT1* transcript to phloem tissue in both roots and shoots suggested that HKT1 is involved in re-circulation of Na from shoots to roots via the phloem. Whether HKT1 can indeed function in both phloem loading (in shoots) and unloading (in roots) as suggested by the authors still requires more evidence, as such dual function depends on opposite electrochemical gradients between phloem and adjacent cells in the two tissues. Based on the kinetic analysis of ^{22}Na fluxes a function of HKT1 in Na re-circulation has recently been challenged (Essah et al., 2003).

Increasing evidence points to so-called voltage-independent channels (VICs) or nonselective cation channels (NSCCs) as the major pathway for Na entering the cytoplasm. The open probability of this channel type does not depend on voltage (Amtmann and Sanders, 1999; Demidchik et al., 2002; Maathuis and Sanders, 1999; Tyerman, 2002; White, 1996), and therefore changes of current through VICs in response to changes of voltage are instantaneous. VICs in barley, maize and wheat

discriminate only weakly between K and Na and were therefore suggested to be the main pathway for Na uptake into plants (Amtmann and Sanders, 1999). This is supported by the coincidence that both currents through VICs and Na fluxes into plant roots are inhibited by external Ca (Davenport and Tester, 2000; Tyerman and Skerrett, 1999). In Arabidopsis VICs were also found to be inhibited by cyclic nucleotides, which agrees with the finding that the addition of cyclic nucleotides partially alleviated salt-stress symptoms of Arabidopsis seedlings (Maathuis and Sanders, 2001). A recent study with *Thellungiella halophila*, a salt-tolerant close relative to *A. thaliana* further strengthened the role of VICs in salt stress. VICs in root cells of Thellungiella had higher selectivity for K over Na than the respective channels in Arabidopsis (Volkov et al., 2004). However the genes corresponding to VICs remain to be discovered. So-called cyclic-nucleotide gated channels (CNGCs) are voltage-independent and non-selective in animals and this channel type is represented by a large gene family in Arabidopsis (20 members). AtCNGC1 is permeable for Na, but it is an inward rectifier which means its open probability is voltage-dependent (Leng et al., 1999). Further more, cyclic nucleotides activated AtCNGC1 but inhibited root VICs (Maathuis and Sanders, 2001). Therefore it is unlikely that AtCNGC1 is responsible for the instantaneous Na currents observed in Arabidopsis root protoplasts. Another member of the Arabidopsis CNGC family, AtCNGC4 was identified as a non-selective VIC in *Xenopus* oocytes but resides in leaves where it is involved in the hypersensitive response (Balague et al., 2003). AtCNGC2 was found to be voltage-independent too but it is K-selective (Leng et al., 2002), and therefore unlikely to be responsible for Na uptake. CNGC3 presents at the plasma membrane of root cortical and epidermal cells and ubiquitously in shoot tissues in Arabidopsis. The function of CNGC3 is likely to be non-selective uptake of monovalent cations in Arabidopsis root (Gobert et al., 2006). Whether other members of

the CNGC family could be responsible for Na uptake into plant cells remains an open question.

A low-affinity cation transporter (LCT1) cloned from wheat has no homology to any known membrane transporter. When expressed in yeast, LCT1 mediates the uptake of K (Rb), Ca, Na and heavy metals (Clemens et al., 1998; Schachtman et al., 1997). Expression of LCT1 increased intracellular Na levels and salt sensitivity of a salt-sensitive yeast mutant deleted in the Na-export pump *ENA1*. This phenotype was alleviated by other cations, including K and Ca, through competitive inhibition (Amtmann et al., 2001). The increased sensitivity of LCT1 transformed yeast to high concentration of Ca suggests that Na and Ca transport in plants could be mediated by the same transporter. In contrast to VICs, Na transport through LCT1 in the millimolar range is not affected by micromolar Ca concentrations. The main function of LCT1 in plants might be the uptake of Ca, but under salt stress, LCT1 could be an important pathway for Na uptake. Unfortunately, functional characterization of LCT1 *in planta* has not yet been done and its tissue expression is unknown.

Unlike yeast and bacteria, plants do not possess Na pumps that are directly energized by ATP. However proton-coupled Na transport in both plasma and tonoplast membrane has been well established in many higher plant species (Barkla and Pantoja, 1996; Mennen et al., 1990; Wilson and Shannon, 1995). A plasma membrane Na/H antiporter, *SOS1* (*AtNHX7*) was identified as the locus controlling the salt-sensitivity of the salt-oversensitive Arabidopsis mutant *sos1* (Shi et al., 2000; Wu et al., 1996). *SOS1* belongs to a large gene family (8 *NHX* genes in Arabidopsis genome) that is closely related to Na/H antiporters of bacteria and fungi. The protein encoded by SOS1 is predicted to have 12 transmembrane-spanning domains and a long C-terminus in the cytoplasm which might interact with various regulators (e.g. the Ca-dependent SOS2-SOS3

complex, Shi et al., 2000). SOS1 is localized to the plasma membrane and is strongly expressed in root xylem parenchyma cells (Shi et al., 2002). Arabidopsis plants overexpressing SOS1 display increased salt tolerance both during seedling development and as mature plants, and show reduced Na-accumulation in shoots (Shi et al., 2003). A function of SOS1 in both delivery and retrieval of Na to/from the xylem has been proposed (Shi et al., 2002; Tester and Davenport, 2003).

Another gene of the Arabidopsis NHX family, AtNHX1, is located in the tonoplast and plays an important role in compartmentalizing Na into the vacuole through electroneutral Na/H exchange (Apse et al., 1999). As for SOS1, a topology of 12 transmembrane-spanning domains and a long C-terminus is predicted from the sequence of NHX1. The N-terminus is located in the cytoplasm and the C-terminus in the vacuole (Yamaguchi et al., 2003). Interestingly, truncation of the C-terminus differentially affected transport rates of Na/H and K/H, thus indicating that the substrate-specificity of NHX1 is regulated from the vacuolar side (Yamaguchi et al., 2003). Over-expression of AtNHX1 in Arabidopsis as well as *Brassica napus* and *Lycopersicon esculentum* increases salt tolerance of mature plants (Apse et al., 1999; Zhang and Blumwald, 2001; Zhang et al., 2001). Over-expression of AtNHX1 in tomato enhances salt accumulation in leaves without affecting Na levels in fruits or lipid composition of seeds. *NHX2* and *NHX5* were identified from Arabidopsis on the basis of sequence similarity to *NHX1* and analyzed with respect to Na transport and transcriptional regulation by salt (Yokoi et al., 2002). Like *NHX1, NHX2* is strongly expressed in both roots and shoots and localizes to the tonoplast. *NHX5* transcripts are also present in roots and shoots but at a lower level. They both suppress the Na/Li-sensitive phenotype of a yeast mutant defective in the vacuolar antiporter *ScNHX1* (Yokoi et al., 2002), but differ in transcriptional regulation. Whereas *NHX2*, similar to *NHX1*, is up-regulated by osmotic

stress (Na and sorbitol) in an ABA-dependent manner, *NHX5* responds only to ionic stress (Na) and is independent of ABA (Yokoi et al., 2002). In summary, it appears that NHX-type transporters in Arabidopsis are involved in active Na extrusion from the cytoplasm but differ in membrane location and regulation.

Long distance transfer of Na and tissue specific control of Na transport is important for whole-plant Na homeostasis. Na accumulation in photosynthetic tissue inhibits photosynthesis. Many salt tolerant species and salt tolerant varieties of glycophytic species have lower net accumulation of salt in leaves (Munns, 2002). Reduced Na transfer from the root to the shoot or enhanced Na recirculation from the shoot to the root, or both, limit Na accumulation in the shoots. This can be achieved by (i) decreasing root xylem loading, (ii) increasing retrieval from the root xylem, (iii) decreasing xylem unloading in the shoots and (iv) increasing phloem loading in the shoots. As mentioned above, HKT1 might be involved in recirculation of Na from shoots to roots via the phloem, and SOS1 might function in both delivery and retrieval of Na to/from the xylem. Together with SOS1, the outward-rectifier SKOR1 and a putative cation-proton cotransporter (CHX21) provide further pathways for cation delivery to the root xylem (Gaymard et al., 1998; Hall et al., 2006). To gain further insight into the functional differentiation between these three transporters, subtissue and multiple knockouts are required.

Since the discovery of Na/H antiporters and the SOS signalling pathway in Arabidopsis, Na export has been the focus of research interest in plant salt tolerance. However, more physiological studies are required to define the main strategy adopted by salt tolerant plants to control Na uptake. Only under the guidance of meaningful physiological data, can molecular identity and control of ion transport be addressed more efficiently.

1.3.1.2 Interaction between Na and other cations

Potassium is essential to all plant life, and in most terrestrial plants K is the major cationic inorganic nutrient (Marschner, 1995). In a high salt environment, because of the similar physicochemical structures of Na and K, Na competes for K entry into the symplast, which may result in K deficiency (Maathuis and Amtmann, 1999). In particular, high concentrations of Na can interfere with high-affinity K uptake. For example, high-affinity K transport through a wheat K-Na symporter, TaHKT1, is replaced by low-affinity Na uptake at high external Na concentration (Gassmann et al., 1996; Rubio et al., 1999). Net loss of K may result from membrane depolarization when external Na concentration increases. Furthermore, cytoplasmic Na competes for K binding sites on enzymes and hence inhibits metabolic processes that crucially depend on K (Greenway and Munns, 1980). Consequently the Na/ K ratio in the cytosol is considered a more critical factor in determining Na toxicity, than the cytosolic Na concentration *per se* (Maathuis and Amtmann, 1999).

Calcium is an essential plant nutrient. Ca is required for the structural integrity of the cell wall and membranes, as a counter-cation for inorganic and organic anions in the vacuole and as an intracellular messenger in the cytosol (Marschner, 1995). During salt stress Na replaces plasma-membrane-bound Ca, resulting in decreased mechanical stability of the plasma membrane (Lauchli, 1990b). A high Na/Ca ratio or low concentration of Ca *per se* are suggested to increase membrane permeability (Lauchli, 1990b). Application of external Ca has long been known to ameliorate salinity stress symptoms in many species (Cramer et al., 1989; Elphick et al., 2001; Lahaye and Epstein, 1969; Martinez and Lauchli, 1993). One reason for this could be that Ca and Na share common transport pathways. Thus in a salt-sensitive yeast strain expressing TaLCT1 additional Ca decreased intracellular Na accumulation and salt hypersensitivity

through competitive inhibition of Na uptake by this transporter (Amtmann et al., 2001). Furthermore, Ca is involved as a secondary messenger in signalling cascades that modify the activity of ion transporters. For example, an increased Ca concentration in the culture medium suppresses the Na-hypersensitivity of the Arabidopsis mutant *sos3* (Liu and Zhu, 1998). This phenotype is due to the fact that the plasma membrane Na/H antiporter SOS1 is activated by a protein complex formed between the Ca-dependent protein kinase SOS3 and the Ca-binding protein SOS2 (Shi et al., 2000). Direct inhibition of VIC (voltage independent channel)-mediated Na currents by micromolar external Ca has been demonstrated in wheat and maize root protoplasts (Davenport et al., 1997; Roberts and Tester, 1997b; Tyerman and Skerrett, 1999; Tyerman et al., 1997). Ca also sustains K transport and K/Na selectivity in salt-stressed plants but how this is achieved is still largely unknown (Lauchli, 1990a).

1.3.2 Osmotic Adjustment

Osmotic shock caused by a sudden increase of salinity triggers a rapid and transient reduction in rates of leaf expansion and root elongation. This response is shown by a wide range of species, and also occurs when other osmotica such as KCl, mannitol or polyethylene glycol (PEG) are applied. It is therefore not specific for NaCl stress, but due to changes in cell water relations. Passioura and Munns (2000) confirmed this hypothesis with pressurization experiments. Wheat and barley plants were kept under maximum pressure in a pressure chamber when changes of salinity were applied. The absence of changes in water pressure prevented the transient growth reduction in the early stage of salt stress (Passioura and Munns, 2000). In addition, pressurization also prevented the steady state growth reduction during the early stage of salt stress. Therefore, salt stress and drought share common signalling pathways and downstream responses (Zhu, 2002).

Increased salt concentrations make the water potential of the soil more negative than the root symplast, resulting in tissue dehydration. The plant root must establish a water potential gradient so that water flows into the plant from the soil. Plants can regulate their solute (osmotic) potentials within a certain range to compensate for the low external water potential, and this is called osmotic adjustment (Bray et al., 2000).

During the early stages of salt stress Na taken up by roots can act as an osmoticum to help restore turgor when compartmentalized in the vacuole without increasing cytosolic Na concentration (see above). If salt stress persists, plants accumulate organic solutes including primarily organic acids, nitrogen compounds and carbohydrates, e.g. malate, aspartate, glutamate, glycinebetaine, proline and sucrose, in the cytoplasm to maintain a low water potential in the cell (Greenway and Munns, 1980). Some of these non-inhibitory metabolites may aid osmotic adjustment between the cytoplasm and the vacuole while other organic acids (e.g. malic acid) may balance excess cation levels within the vacuole. It has been suggested that organic solutes in the cytoplasm have two major roles: contributing to the osmotic balance when electrolytes are lower in the cytoplasm than in the vacuole, and protecting enzyme structure in the presence of high concentrations of electrolytes in the cytoplasm (Rontein et al., 2002). Therefore apart from osmotic adjustment, accumulation of compatible organic solutes under high salt concentration, also plays an important role in damage protection and detoxification.

1.3.3 Detoxification and Growth Regulation

Although ion uptake may provide a means for osmotic adjustment, high cytosolic concentrations of Na and/or Cl ions are toxic to the cell by the adverse effect on cell membrane integrity, enzyme activity, nutrient acquisition and function of the photosynthetic apparatus (Munns, 1993).

Several protein kinases including mitogen-activated kinases activated by osmotic stress may mediate detoxification responses (Droillard et al., 2000; Gustin et al., 1998; Jonak et al., 1996; Kovtun et al., 2000; Mikolajczyk et al., 2000; Seo et al., 1995). The MAP kinase cascade is activated by hyper-osmotic conditions and other stresses. Currently, the functional significance and the output of the kinase activation are unclear.

Most of the changes in gene expression induced by salt stress can be considered as part of detoxification signalling. These include changes in the expression of LEA/dehydrin-type genes (Xiong et al., 2001a; Xiong et al., 2001b; Zhu, 2001a; Zhu, 2001b), molecular chaperones and proteinases that remove denatured proteins (Krochko et al., 1998; Takahashi et al., 2001; Xiong et al., 2001b), enzymes involved in the generation and removal of reactive oxygen species and other detoxification proteins (Allen et al., 1997; Roxas et al., 1997; Sang et al., 2001; Tsugane et al., 1999; Zhu et al., 1997), as well as enzymes involved in phospholipid hydrolysis. For example, phospholipid signalling involves several types of phospholipases that cleave phospholipids to generate lipid messengers (e.g., PA, DAG and IP_3), which regulate stress tolerance partly through modulation of stress-responsive gene expression (DeWald et al., 2001; Drobak and Watkins, 2000; English, 1996; Heilmann et al., 1999; Hirayama et al., 1995; Munnik et al., 1998; Sang et al., 2001; Takahashi et al., 2001). This pathway can be activated by salt stress, drought, cold, or abscisic acid (ABA).

1.4 Engineering salt tolerance in crops

Salt tolerant plants (halophytes), and variation in salt tolerance between genotypes within glycophytic species provide a genetic basis for engineering salt-tolerant crops (Epstein, 1977). Stress responses and tolerance strategies of plants to salinity have been extensively studied for decades. The ultimate goal of all the studies is to engineer salt tolerant crops and produce economically valuable species. Traditional breeding

approaches alone have not been greatly successful so far in producing salt-tolerant crop varieties due to the genetic and physiological complexity of salt tolerance. The progress in improving salt tolerance is likely to be accelerated by applying current biotechnological techniques. To produce salt-tolerant crop species requires a combination of studies of halophytic species (Chauhan et al., 2000; Flowers et al., 1977; Very et al., 1998), comparative analysis of crop varieties that differ in salt tolerance (Davenport et al., 2005; Munns et al., 2000), and mutants screening in Arabidopsis (Zhu, 2000).

1.4.1 Modernizing the traditional breeding approach

Enhancing salt resistance of 'at least some crops' by conventional breeding has been encouraged by Flowers and Yeo (1995). But this approach has encountered several problems. One factor limiting the progress is the complexity of the salt tolerance trait, which is controlled by a number of genes or groups of genes, and involves a number of component traits which are likely to be quantitative in nature. Another limiting factor might be found in a lack of communication between plant scientists and plant breeders during the last century (Flowers and Yeo, 1995). This situation has changed dramatically over the last few years. Especially in China, breeding institutions (e.g. China National Hybrid Rice R&D Centre) work closely together with researchers at the universities. The recent development of high throughput techniques for the molecular analysis of plant material (e.g. microarrays and metabolite profiling) are likely to revolutionize breeding strategies and lead to large-scale genetic and 'metabolic engineering', involving the modification of many genes (Bohnert and Jensen, 1996). Therefore modern plant breeding is no longer a time-consuming simple selection process, but a multi-disciplinary effort to transform traits developed in molecular biology laboratories into reliable new crop lines in a farmer's field.

1.4.2 Salt-adapted cell lines

Selection of tissue culture or cell culture for regeneration of salt tolerant plants was adopted as an alternative to simple selection of salt tolerant varieties. According to the review by Chandler and Thorpe (1986), induced genetic variation can be inherent to both plant cell cultures and regenerated plants from the cultured cells. Cultured cells are amenable to large scale selection and mutagen treatment prior to selection is not necessary, therefore using cell cultures to select for salt tolerance *in vitro* has its obvious advantages (Chandler and Thorpe, 1986).

A wide range of species have been grown in tissue or cell culture to select for salt tolerance, including the well established tobacco model system (Binzel et al., 1985; Heyser and Nabors, 1981a; Heyser and Nabors, 1981b; Larosa et al., 1985; Pua and Thorpe, 1986) and several important crop species, such as oat, *Sorghum*, *Brassica* spp., rice, alfalfa, *Citrus* spp., sugar beet and tomato (reviewed by Chandler and Thorpe, 1986). A suspension cultured salt-resistant Arabidopsis cell line has been developed at Glasgow University, UK (Price, 2005). The HHS (Habituated to High Salt) Arabidopsis cell line was developed from the WT line, by successive weekly transfer from a 50 mM NaCl 'seed culture' into progressively higher NaCl concentrations. Over a period of 4 years, this cell line was successfully habituated so that it now grows well in 300 mM NaCl (Price, 2005).

The disadvantage of this approach is that selects for salinity resistance at the cellular level, and the selected traits might not confer salt tolerance to the whole plant. Tissue specific traits and those linked to certain developmental stages will not be discovered in cell cultures. Furthermore, it is impossible *in vitro* to accurately mimic soil-root or plant-environment interactions or the complex differences between soils in structure, composition and ion interaction (Chandler and Thorpe, 1986). It is important to

remember that the goal of selection is to produce salt-tolerant plants but not obligatory halophytes, and this should be achieved without a trade-off in yield or desirable characteristics such as disease resistance (Chandler and Thorpe, 1986). Nevertheless the cell culture should help to reveal the successful adaptative mechanisms of ion transport and metabolic regulation to high salinity at the cellular level.

1.4.3 The study of halophytic plants

Halophytes are plants which complete their life cycle in conditions of high salinity (Flowers et al., 1977). Studies of the mechanisms by which naturally occurring halophytic plants cope with salt stress are widely conducted. In theory, this approach should be more productive than studies on salt-induced damage or 'panic responses' in glycophytes. Knowledge of halophytic strategies for coping with salinity was considered fundamental to any attempt to develop crops combining adequate tolerance with yield (Flowers et al., 1977). Although physiological experiments including measurements of ion contents, organic osmotica and enzyme activities have been carried out for a number of halophytic species, e.g. *Suaeda* spp., *Atriplex* spp., *Avicennia marina*, *Aster tripolium*, *Salicornia fruticosa*, *Triglochin maritima* and *Mesembryanthemum crystallinum*, only *M. crystallinum* has been developed into a model plant for analyzing molecular features such as expression and regulation of ion transporters, stress signalling events and metabolic pathways (Thomas and Bohnert, 1993).

M. crystallinum possesses bladder cells which gave it the name 'ice plant'. Its leaves are covered with large epidermal bladder cells, giving them a distinctive glistening appearance. These bladder cells store large quantities of NaCl and somatically balance the amount of polyols, which allows the plant to be a salt-accumulator and conserve water at the same time (Adams et al., 1992). Another distinct feature of this plant is a

developmentally programmed switch from C_3 photosynthesis to Crassulacean Acid Metabolism (CAM) which is accelerated by salinity and drought (Adams et al., 1998). CAM minimizes water loss and ensures reproductive success in the absence of rain and in saline soils (Winter, 1985). Unfortunately no crop species possesses either of the two features. Neither genetic engineering of a complex morphological organ nor the introduction of a distinct metabolic pathway into another species are realistic goals for genetic engineering. Therefore the value of studies with *M. crystallinum* for the development of salt-tolerant crops is limited.

Recently, *Thellungiella halophila* has been established as a new model species for research into plant salt tolerance (Amtmann et al., 2005; Bressan et al., 2001; Zhu, 2001b). *T. halophila* (from now on referred to as Thellungiella) is closely related to *Arabidopsis thaliana* (from now on referred to as Arabidopsis) but shows considerably higher tolerance towards abiotic stresses including salinity, cold and drought (Bressan et al., 2001; Zhu, 2001b; Amtmann et al., 2005). Despite the striking difference in stress physiology Thellungiella and Arabidopsis share fundamental morphological and developmental features, and are both C_3 plants. Together with high homology at the DNA level these characteristics suggest that the Thellungiella/Arabidopsis pair represents not only a good model system but also a potential genetic resource for the engineering of salt tolerant crops. The work presented in this thesis is based on a comparative analysis of salt tolerance in Thellungiella and Arabidopsis.

1.4.4 Comparative studies of crop varieties with different level of salt tolerances

Sequencing of the rice genome (International Rice Genome Sequencing Project) was completed at the end of 2002 and the annotation work is ongoing. It will provide genomic information and molecular tools for rice research (website

http://rgp.dna.affrc.go.jp/IRGSP/). Screening of the large germplasm stocks of important crops was organized by agencies such as the Food and Agricultural Organization of the UN and have led to the identification of salt tolerant varieties of wheat and barley (Epstein et al., 1980) and rice (Ponnamperuma, 1982). Therefore studies of crop species with different levels of salt tolerance have become feasible.

Extensive work has been done in this field. Data are available not only on physiological characteristics such as growth and/or ion homeostasis in response to salt in varieties of wheat (Davenport et al., 2005; Jbir et al., 2001; Santa-Maria and Epstein, 2001; Watson et al., 2001), barley (Garthwaite et al., 2005) and rice (Lee et al., 2003), but also on molecular characteristics of specific genes (e.g. AKT1-like K channel in rice, (Golldack et al., 2003); O-methyltransferase in wheat, (Sugimoto et al., 2003)) and salt induced profiles of the transcriptome (e.g. in barley, (Ozturk et al., 2002)) and the proteome (e.g. in wheat, (Majoul et al., 2000; Ouerghi et al., 2000) and rice (Parker et al., 2006)). However most crop species have larger genomes and lack sequence information. Investigating molecular mechanisms of salt tolerance in these species will continue to be a difficult task.

1.4.5 Mutant generation

A screening project by J. K. Zhu and colleagues at the University of Arizona used a root-bending assay with *in vitro* growing Arabidopsis plants to select for mutants that were hypersensitive to salt stress (Wu et al., 1996). A large genetic screen for Salt Overly Sensitive (*sos*) mutants (Shi et al., 2000; Zhu et al., 1998) has revealed the Ca-dependent SOS signalling pathway which regulates the plasma membrane Na/H antiporter, SOS1, in Arabidopsis. Salt tolerant lines of Arabidopsis have been generated by over expressing SOS1 (Shi et al., 2003). Thus screening for mutants with impaired

salt tolerance proved to be an efficient approach to identify genes and cellular processes crucial for plant responses to salt stress.

Instead of screening for salt hypersensitivity, Price (2005) searched for increased salt tolerance in Arabidopsis activation-tagged lines (Weigel et al., 2000). This large-scale gain-of-function screen has already identified several single sequences that allow seedlings to survive better under NaCl stress. Although salt tolerance of plants can alter with developmental stage, this novel screening approach has the potential to reveal key genes facilitating salt tolerance.

Lahner *et al.* (2003) screened Arabidopsis mutants for abnormal ion accumulation using ICP-MS. They estimated that 2 to 4% of the Arabidopsis genome is involved in regulating plant nutrient and trace element composition. Their study demonstrated the utility of elemental profiling as a functional genomics tool (Lahner et al., 2003).

Despite their scientific merits all the above studies might be limited in their value for developing salt tolerant plants by the fact that certain crucial functional components of salt tolerance that were lost during evolution cannot be restored by mutation of the existing Arabidopsis genome.

1.5 Aim of the thesis

This thesis addresses the question of how salt tolerant plants control ion transport in response to salt stress. To answer this, a series of comparative studies were carried out with two closely related model species, salt-sensitive *Arabidopsis thaliana* and salt-tolerant *Thellungiella halophila* (Figure 1-1). Ion transport in particular Na transport under salt stress was analysed using ^{22}Na tracer flux analysis and ICP-OES. Salt induced transcriptional regulation of ion transporters was compared between the two species using DNA microarrays.

1.5.1 Model system

The advantage of this study is the model system consisting of two closely related plants with different levels of salt tolerance.

1.5.1.1 Arabidopsis thaliana

Arabidopsis thaliana, a modest little flowering *Brassica* species related to broccoli and cauliflower, is *the* model plant (Dennis and Surridge, 2000). Its small size, short life cycle and prodigious seed production make it an ideal organism to propagate in the laboratory. And with a relatively small genome of about 120 Mb it is perfect for sequencing. Arabidopsis contains a complete set of genes for controlling developmental patterns, metabolism, responses to environmental stimuli and disease resistance, without much of the repetitive DNA present in the genomes of other higher plants. Thus its genomic sequence provides a means for analyzing gene functions relevant to a range of plant species, including commercially important crops.

The entire genome of Arabidopsis has been sequenced (Dennis and Surridge, 2000) and the data can be easily accessed on the Internet (e.g. http://www.arabidopsis.org/ and http://mips.gsf.de/proj/thal/). In the USA, the Arabidopsis Functional Genomics Consortium (AFGC) (http://afgc.standord.edu) was established to coordinate the study of gene function by two synergistic methods, microarray gene expression profiling and gene knockout mutagenesis. In the UK, GARNet (http://garnet.arabidopsis. org.uk/) supports researchers in a similar effort. Useful functional genomics tools, e.g. microarrays covering the whole genome are available for academic use (http://www.affymetrix.com/ and http://ag.arizona.edu/microarray/).

1.5.1.2 Thellungiella halophila

Thellungiella halophila (salt cress), synonymous to *T. salsuginea* (Al-Shehbaz et al., 1999), is a close relative of Arabidopsis. It is a real extremophile that is tolerant to salinity, drought and cold, The Shandong ecotype used for this study is native to the seashore saline soils of eastern China but ecotypes from other parts of the world have also been collected (Amtmann et al., 2005). Although Thellungiella shares a similar morphology and life history, having been confused with Arabidopsis in the past (Al-Shehbaz *et al.*, 1999), it is not in the Arabidopsis genus, and with seven chromosomes, cannot be crossed successfully with Arabidopsis. Its genome size is less than twice that of Arabidopsis. EST analyses of several hundred Thellungiella clones revealed averages of 90% and 95% identities between salt cress and Arabidopsis cDNA and amino acid sequences respectively (Bressan et al., 2001). Thellungiella is reported to be able to tolerate shock treatment of up to 500 mM NaCl (Bressan et al., 2001). Since it does not produce salt glands or other complex morphological alterations either before or after salt adaptations, salt tolerance in Thellungiella appears to be largely the result of basic biochemical and physiological mechanisms that can be subject to individual gene mutations (Bressan et al., 2001).

Genotypic and phenotypic similarity with Arabidopsis (Volkov et al., 2004; Inan et al., 2004) will facilitate the identification and cloning of Thellungiella genes and their over-expression in Arabidopsis will provide an unprecedented opportunity for functional analysis of putative salt tolerance genes in a highly similar glycophytic background. Within the last few years several molecular tools have been created for Thellungiella including collections of ESTs, T-DNA insertion mutants and ecotypes as well as cDNA libraries and microarrays (Amtmann et al., 2005, http://thellungiella.org/).

A comprarison between Arabidopsis and Thellungiella has a definite advantage over previous comparisons between glycophytic and halophytic plants as it can utilize the molecular resources and functional genomics tools of Arabidopsis. However, to determine which specific salt tolerance traits should be characterized at the molecular level basic physiological parameters required comparative quantification, including growth, transpiration, ion uptake, accumulation and tissue allocation, membrane potential and currents.

1.5.2 Experimental strategies

Arabidopsis and Thellungiella plants were grown hydroponically in controlled growth chambers. Salt stress was applied by supplying the growth solution with additional NaCl. In this way salt treatment is given homogeneously and more quantitatively than watering soil-grown plants with salty water. The amount of other nutrients supplied to the plants is controlled, and as a result ion content profiles are more comparable between plants and plant batches. The accumulation of Na and several nutrient elements in shoots and roots of Arabidopsis and Thellungiella plants subjected to short term and long term salt stress was analyzed with Inductively Coupled Plasma – Optical Emission Spectroscopy (ICP-OES). Kinetics of net Na accumulation and unidirectional Na fluxes were characterized in both species with ICP-OES and ^{22}Na tracer flux technique respectively. Finally, salt dependent expression patterns of genes encoding known and putative ion transporters in the two species were compared using microarray technology.

1.6 Outline of the thesis

Following this Introduction, four chapters are presented including three Results chapters and one general Discussion chapter. These are: Chapter 2: Na accumulation and fluxes, Chapter 3: Homeostasis of other ions under salt stress, Chapter 4: Transcriptional

profiles and Chapter 5: Conclusions and Outlook. Each Results chapter has four parts: Introduction, Materials and Methods, Results and Discussion. Chapter 5 combines all the results produced in this thesis and discusses them together with results from electrophysiological studies carried out by Dr. Vadim Volkov in the same laboratory. Conclusions drawn from the data with respect to mechanisms underlying salt tolerance in Thellungiella and some ideas for future research in this field are also presented in Chapter 5.

Figure 1-1. The two closely related model species, salt-sensitive *Arabidopsis thaliana* (left) and salt-tolerant *Thellungiella halophila* (right).

Chapter 2 Na accumulation and fluxes

2.1 Introduction

This chapter presents a comparative analysis of Na uptake into roots of Thellungiella and Arabidopsis. It covers net Na accumulation after short- and long-term salt stress, kinetics of net Na uptake over 3 days of salt treatment and steady-state unidirectional Na fluxes into and out of roots of Thellungiella and Arabidopsis. Furthermore, unidirectional Na influx is compared with net Na uptake to determine the contributions of Na influx and efflux across the root plasma membrane to the total net Na uptake by Arabidopsis and Thellungiella plants.

I will give a brief technical introduction into ^{22}Na tracer flux experiments and review existing knowledge on Na fluxes in Arabidopsis and other species. The experiment set up will be summarized and the questions addressed will be outlined. Technical information about ICP-OES technology will be explained in the Introduction of Chapter 3.

2.1.1 Radioactive tracer fluxes

The isotopic tracer technique is widely used to measure unidirectional fluxes of specific ions across biological membranes. Following the movement of radioactive Na (e.g. ^{22}Na) allows us to distinguish the unidirectional influx of Na from its net accumulation (sum of root influx, efflux and re-circulation) and apoplastic bypass, because at the beginning of the experiment radioactive Na is only present in the external medium. Thus, although Na moves in both directions the radioactivity reflects only inward movement. As ^{22}Na builds up inside the cell, efflux of ^{22}Na gradually increases and then net influx can be measured. It is essential to establish the time course of ^{22}Na movement

into roots to determine the initial linear component that accurately reflects unidirectional influx.

In cells and/or tissues where compartments exist in series, the Na flux across a particular membrane can be determined by fitting the kinetics of ^{22}Na movements with separate kinetic components according to a spatial model of cell and/or tissue compartments (MacRobbie, 1981b). By applying various putative blockers and agonists, we can distinguish between possible ion transport pathways.

Detailed characterization of unidirectional Na influx has been conducted in cereals such as wheat (Allen et al., 1995; Davenport et al., 2005; Davenport et al., 1997; Davenport and Tester, 2000) and maize (Zidan et al., 1991), and the non-cereal Arabidopsis (Essah et al., 2003; Maathuis and Sanders, 2001).

2.1.2 Technical improvement in this study

Several previous studies with Arabidopsis have used seedlings grown in petri dishes or in suspension cultures to measure Na influx (Essah et al., 2003; Maathuis and Sanders, 2001). Because of the high humidity in petri dishes, plants grown in petri dishes have very low transpiration rate. In suspension culture, transpiration is completely absent and Na might be taken up through transporters in the shoots that do not normally contribute to Na influx.

Most ^{22}Na flux studies on mature plants have been conducted with the excised root system (Davenport et al., 1996; Davenport and Tester, 2000; Essah et al., 2003; Zidan et al., 1991). In this system the delivery of ions such as Na to root cells relies on passive diffusion. Ion uptake by epidermal and outer cortical cells is determined by the availability and mobility of ions in the external solution, which might be limited by reduced bulk flow to the stele in excised roots. This could affect the apparent kinetics of ion uptake, and also reduce the contribution of epidermal and outer cortical cells to

uptake. In this study we measured Na fluxes into roots of living mature and transpiring plants grown in liquid nutrient solution. The results are expected to reflect the physiological level of Na influx into the plants in a natural environment.

A standard equilibration (pre-treatment) step with an unlabelled solution of the same concentrations and chemical composition as the ^{22}Na labelled influx solution was adopted in this study to adapt the plant to the chemical composition of the labelling solution. In this way, Na influx into the roots was measured in steady-state.

2.1.3 Na influx into roots of Arabidopsis and other plant species

Essah et al (2003) characterized unidirectional Na influx extensively in excised roots from seedlings of wild type Arabidopsis and various mutants. In a ^{22}Na labelled solution with 200 mM NaCl, ^{22}Na levels in the excised roots of wild type Arabidopsis increased linearly in the first 2 min, and this increase slowed down significantly after 5 min. The linear Na influx component was about 3.3 μmol/g FW/min at a Ca activity of 0.2 mM. This rate was sensitive to the external Ca concentration and treatment with DEPC (diethylpyrocarbonate), a reagent that modifies His and Tyr residuals in proteins (Mankelow and Henderson, 2001; Row and Gray, 2001). Unidirectional Na influx was inhibited by 3 mM external Ca activity to about one-third of the level in 0.05 mM external Ca activity. Ca inhibition was found to be sensitive to hyperosmotic pre-treatment. Treatment with sorbitol decreased Na influx in low external Ca, without further reduction in higher Ca concentration. The authors suggest that hyperosmotical inhibition of Ca sensitivity might be due to a rise in cytosolic Ca concentration induced by the change in osmotic potential. The Ca sensitivity of Na influx was not due to Na uptake through a Ca channel since verapamil, a Ca channel blocker, had no effect on Na influx.

With respect to the transporters involved in Na uptake, the following conclusions were made by Essah and colleagues (2003): 1) Na influx into roots of Arabidopsis is not through a Na/H antiporter, because treatment with amiloride, an inhibitor of Na/H antiporters had no effect on Na influx, and Na influx into roots of the Na/H antiporter knockout mutant *sos3-1* was similar to the wild type control. 2) Na influx is not through shaker-type K channels, because K channel knockout mutants, *akt1* and *skor1*, showed no significant change in Na influx into the roots. Furthermore, treatments with Cs and TEA (blockers of K inward and outward rectifying channels respectively) did not alter Na influx in wild type Arabidopsis. 3) Na influx is not through HKT1, a Na transporter in Arabidopsis, because the knockout mutant *hkt1-3* did not show altered Na influx compared with wild type Arabidopsis.

There is increasing evidence that voltage-independent nonselective cation channels (NSCC or VIC) are the major pathway for Na entry into root cells. Essah et al. (2003) found that treatments with flufenamate and quinine, (NSCC blockers in animals), reduced Na influx into roots of Arabidoopsis. In contrast, addition of glutamate increased Na influx, which could indicate that members of the glutamate receptor gene family are involved in Na uptake. Cyclic nucleotides, e.g. membrane-permeable analogs of cGMP and cAMP treatments also inhibited Na influx suggesting a role of cyclic nucleotide gated channels in Na uptake. Similar results had previously been obtained by Maathius and Sanders (2001) for Arabidopsis seedlings grown in suspension culture. They showed that Na influx into seedlings decreased by up to 40% when between 10 to 100 μM cGMP was added, and by up to 30% when between 10 to 500 μM cAMP was present in the solution (50 mM NaCl).

Studies of ^{22}Na influx into root segments of bread wheat (*Triticum aestivum*) also indicated that nonselective ion channels are involved in Na influx (Davenport and

Tester, 2000). Na influx into wheat root segments was inhibited by external Ca and Mg with a K_i of 0.61 and 0.56 mM respectively. The kinetics of this inhibitory effect resembled those of Ca/Mg inhibition of a weakly voltage-dependent nonselective cation channel identified by patch clamp. None of the other blockers that were tested including TEA , verapamil, quinine, amiloride and flufenamate, had a significant effect on Na influx or current through the NSCC.

Another study compared Na fluxes in two lines of durum wheat (*Triticum turgidum*), the relatively salt tolerant landrace line 149 and the salt sensitive cultivar Tamaroi (Davenport et. al., 2005). The most obvious difference between the two varieties is that line 149 has a much lower Na content in the leaf blade after salt stress than Tamaroi. The time course of Na influx into roots and shoots was measured with ^{22}Na tracer flux technique. Na influx and efflux across the plasma membrane and the tonoplast of root cells were calculated by fitting the flux kinetics with a two-compartment model. Trans-plasma membrane Na influx was similar in the two wheat varieties while trans-tonoplast Na influx appeared to be slightly slower in the salt sensitive Tamaroi than the salt tolerant line. However, the largest difference was found in the transfer of Na transport from roots to shoots which was almost 8 times faster in Tamaroi than line 149. This could explain the lower Na accumulation in the leaf blade of the salt tolerant line 149. The Na efflux from roots of salt sensitive Tamaroi was lower in absolute terms compared with line 149, however, the relative efflux expressed as a proportion of total root Na per minute was similar in the two varieties. The authors found no evidence for re-circulation of Na from shoots to roots in this study.

2.1.4 Questions addressed

2.1.4.1 Does Thellungiella tolerate or avoid Na accumulation in the plant?

High Na uptake was previously considered a general property of halophytes (Flowers et al., 1977; Greenway and Munns, 1980). Many halophytes accumulate large concentrations of Na in the vacuoles to maintain turgor pressure for growth. By contrast, most glycophytes respond to salinity with Na exclusion, especially from the leaves. However, it has been recognized that not all halophytes rely on Na accumulation for survival in high salinity. Reduced Na accumulation in photosynthetic tissues is one of the traits related to salt tolerance, in some halophytic species and salt-tolerant varieties of glycophytes (Yeo and Flowers, 1986). As a first approach to reveal the strategies underlying salt tolerance in Thellungiella, I compared tissue Na concentrations in Thellungiella under salt treatment with those in its glycophytic relative Arabidopsis.

2.1.4.2 Is low net Na accumulation in Thellungiella due to low root uptake or high efflux?

Although the tissue Na concentrations showed that Thellungiella accumulates less Na than Arabidopsis, this result is not sufficient to determine whether Thellungiella is a 'salt-extruder' that actively exports Na from the roots, or a 'salt-excluder' that restricts Na uptake into the roots. To determine this, ^{22}Na tracer flux technique was used to dissect net root Na uptake into unidirectional influx and efflux components.

2.1.4.3 Which types of transporters underlie root Na uptake in Thellungiella?

In the search for candidate ion transporters that are responsible for Na influx into Thellungiella roots I carried out a pharmacological characterization of Na influx into the roots of Thellungiella. The results from this, in combination with expression profiling of membrane transporter genes in Chapter 4 and patch clamp studies of ion currents in root

protoplasts by Dr. Vadim Volkov (University of Glasgow), provide fundamental information required for future identification of the genes underlying Na uptake pathways in Arabidopsis and Thellungiella.

2.1.5 Experimental design

2.1.5.1 Plant growth conditions

Plants of Arabidopsis and Thellungiella were grown hydroponically. A minimum nutrient solution (MNS) had been developed previously to grow Arabidopsis (Arteca and Arteca, 2000; Maathuis et al., 2003) and proved sufficient for Thellungiella. The growth conditions were adjusted so that the two species developed similarly, as Thellungiella naturally grows at a slower rate than Arabidopsis. Changing of the growth medium in the boxes allowed fast and homogenous application of ionic treatments. The system enabled easy harvest of clean root material avoiding root damage during removal of soil or other solid support.

2.1.5.2 Measurement of net Na accumulations

In this study a salt concentration that is stressful to Thellungiella, and not too strong for Arabidopsis is required. In most of the experiments 100 mM NaCl was applied. To determine net accumulation of Na, four-week old Arabidopsis and Thellungiella plants were exposed to 100 mM NaCl for various time periods. Ion concentrations were measured in both roots and shoots by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) as this enabled the simultaneous analysis of different cations. Both short-term (25 h) and long-term (6 weeks) responses were studied. The kinetics of net Na uptake after addition of salt were followed over a period of 72 h.

2.1.5.3 Measurement of unidirectional Na fluxes

Unidirectional Na fluxes into and out of the roots of Thellungiella were measured with ^{22}Na tracer flux technique and compared with Na fluxes of Arabidopsis. Considerable effort went into optimising the rinse solution, including variation of rinsing time and composition of the rinse solution. Subsequently, ^{22}Na uptake into roots of Arabidopsis and Thellungiella was followed over a period of time to identify the best time span for measuring the unidirectional influx. This parameter was then compared between the two species and between different conditions (e.g. external Na and Ca concentration, inhibitors). A longer time course of ^{22}Na uptake into both root and shoot was also recorded for Thellungiella so as to provide some information about root to shoot transfer of Na transport in this species. Finally Na efflux from the roots of Thellungiella and Arabidopsis was measured over a period of 4 hours to identify any significant difference in unidirectional Na efflux between the two species.

2.2 Materials and methods

2.2.1 Plant growth

2.2.1.1 Seed surface sterilization

The seeds were surface sterilized before germinating in an agar filled tube according to the following procedure:

1. Seeds were first washed twice with 70% ethanol for 2 min in an Eppendorf tube.
2. The seeds were incubated in bleach (2.5% (v/v) HCl and 0.5% (v/v) Tween 20 in 70% ethanol) for 3 min (Thellungiella) or 5 min (Arabidopsis).
3. The seeds were washed twice in 70% ethanol for 2 min.
4. The seeds were washed 5 times with ddH_2O and left in ddH_2O.

After sterilization, the seeds were kept in the dark at 4°C for at least 3 days before germinating on agar to synchronize the germination time.

2.2.1.2 Germination on agar tubes

A medium with 0.7% (w/v) agar in half strength minimum nutrient solution (see 2.2.1.5) was sterilized by autoclaving. Lids were removed from 1.5 ml Eppendorf tubes. Under a sterile hood the tubes were filled with the warm agar medium and left to dry. One or two sterilized seeds were put on top of each agar tube in the sterile hood. The agar tubes were put into a transparent eppendorf tube box covered with a lid, and the box was wrapped with cling film to prevent water loss. The seeds were left to germinate in a short day growth room (see 2.2.1.4) for 7 to 10 days before the seedlings were ready for hydroponic growth.

2.2.1.3 Hydroponic Transfer

The agar tubes were transferred to 1 litre-boxes containing liquid growth medium when the seedlings had developed 4 rosette leaves. The plastic hydroponic boxes were painted black to prevent algae and fungi growing in the nutrient solution. Holes were drilled into the lids to fit the eppendorf tubes accurately. About 5 mm was cut off from the tip of each agar tube, then the tube was placed into the hydroponics box with the exposed agar emerged in the solution. The hydroponic cultures were maintained in a controlled growth room. The growth solutions were replaced with fresh media weekly.

2.2.1.4 Hydroponic Growth Conditions

Growth conditions were optimized to achieve similar development of Arabidopsis and Thellungiella plants. The long-day growth room for Thellungiella ran a controlled 18 h/6 h day/night cycle with approximately 300 $\mu E/m^2$ light, 22 /18 °C and 60%/70%

humidity. The short day growth room for Arabidopsis ran a 10 h/14 h day/night cycle, with approximately 200 $\mu E/m^2$ light, 22 /18°C and 60%/70% humidity.

2.2.1.5 Hydroponic Growth Solution

A minimal nutrient solution (MNS) was designed from Arteca and Arteca (2000). Concentrated stock solutions of macronutrients were prepared for each mineral component and autoclaved (Table 2-1). The composition of the 1000x micronutrient stock solution is listed in Table 2-2. The growth solution was prepared by diluting all stock components 1000 times in ddH2O. The pH of the media increased from 5.2 to 6 during plant growth.

2.2.2 Treatments and harvesting to determine Na concentration

Arabidopsis and Thellungiella plants were grown hydroponically in MNS for 4 weeks, by which time they had about 18 rosette leaves, before various experimental treatments were applied.

2.2.2.1 Short-term salt treatment

Salt treatment was imposed by changing the growth solution from MNS to MNS with additional 100 mM NaCl ('+NaCl'). The control plants were replenished with fresh MNS ('Control'). Arabidopsis and Thellungiella plants were treated for 25 h in parallel. For both species, each treatment was repeated 4 times and each replicate consisted of 6 to 7 plants pooled for analysis. After treatment, root tissues were excised and quickly rinsed with ice-cold ddH_2O for 30 s, and the shoot tissues were harvested. Fresh weights of both root and shoot tissues were taken immediately after harvesting. The tissue samples were dried at 70°C for 48 hours in an incubator, and dry weights were recorded.

2.2.2.2 Long-term salt treatment

Four week old Arabidopsis and Thellungiella plants grown in MNS were grown in 'Control' and '+NaCl' MNS for another 6 weeks. '+NaCl' medium for Thellungiella contained MNS and 100 mM NaCl, whereas '+NaCl' medium for Arabidopsis contained only 50 mM NaCl. The harvesting procedure was the same as described for the short-term experiment. Four batches of plants were grown and harvested independently as replicates.

2.2.2.3 Time course of net ion uptake

Arabidopsis and Thellungiella plants were grown under 'Control' condition for 4 weeks before application of 100 mM NaCl. Seven to nine plants were harvested individually at 0, 6, 24 and 72 h after the application of salt treatment. The roots were briefly washed in ice-cold ddH_2O, and roots and shoots were separated and weighed immediately. Samples were dried at 70°C for 48 h, and dry weights were recorded.

2.2.3 Ion analysis

2.2.3.1 Ion extraction

2M HCl was added to the dry plant material in 1: 100 (w: v) proportion to the dry weight and incubated overnight at room temperature. Samples were diluted 50 times with ddH_2O and 5 to 10 ml of diluted ion extract was analyzed by ICP- OES.

2.2.3.2 Design of standard solution

The standard solutions were made in 0.04 M HCl which is equivalent to the final concentration of HCl in the ion extractions after dilution. Ion concentrations in the standard solution were selected based on the expected concentrations in the plant material and the detecting limits of the spectrometer. The final concentrations of each

element in the ×1 standard solution are shown in Table 2-3. Six dilutions were used to produce a calibration curve: ×0, ×0.1, ×0.2, ×0.4, ×0.6 and ×1.

2.2.3.3 Measurement of ion concentration

Ion concentrations were determined with an Optical Emission Spectrometer (PerkinElmer Optima 4300DV) controlled by the WinLab32 software package (PerkinElmer Instruments, MA, USA). The standards were measured to create a calibration curve to determine ion concentrations. Then the intensities of the emission at specific wavelengths from the diluted liquid samples were measured and compared with the standard curve, and the concentrations of the corresponding elements were determined. The data out-put from the spectrometer was the concentration of the diluted solution. Element concentrations of plant tissue samples were calculated back to % of DW with Microsoft® EXCEL.

2.2.3.4 Data processing and statistical analysis

In short- and long-term salt treatment experiments, absolute ion concentration was expressed as percent of tissue dry weight (%DW). Relative ion concentrations of the '+NaCl' samples were expressed as percent of the concentration of the 'Control' sample (% control). For the time course of net ion uptake, ion concentration was expressed as mg ion per g dry weight (mg/g DW). For each element, ion concentrations were plotted against time.

To determine the statistical significance of the data from short- and long-term salt treatment experiments, a t test was applied to the comparisons of each ion between Arabidopsis and Thellungiella after the same treatment and to the comparisons of each ion between 'Control' and '+NaCl' samples within each species. Probability (P) values were obtained expressing the likelihood with which two values from 'Control' and

'+NaCl' samples were the same. Time courses of net ion uptake were fitted to various kinetic models with SigmaPlot® (Systat Software Inc., Richmond, USA).

2.2.4 Treatments and measurement of ^{22}Na fluxes

2.2.4.1 Plant growth

Four week old plants of Arabidopsis and Thellungiella were transferred from MNS to MNS with 100 mM NaCl one week before the experiment to achieve steady-state Na uptake during tracer experiments.

2.2.4.2 ^{22}Na influx

The general procedure for ^{22}Na influx experiments included pretreatment, labelling, washing, harvesting and counting. Roots were killed by boiling for 10 min in water in experiments evaluating apoplastic binding. Four plants were measured individually for each sample.

1. Pretreatment: Salt treated plants were pretreated with non-labelled loading solution for 5 min (3 min in channel blockers to minimize toxicity) before labelling. The composition and concentrations of nutrient ions in the non-labelled solution was exactly the same as in the respective ^{22}Na labelled loading solution (Table 2-4).
2. Labelling: Pre-treated plants were labelled in ^{22}Na labelled loading solutions (as stated in Table 2-4) for the indicated time. The plants were gently agitated during labelling on a rotating shaker to provide aeration and reduce boundary layer effects.
3. Washing: After labelling, the roots were quickly dipped in de-ionised H_2O, then washed for 3 min in ice-cold rinse solution (as stated in Table 2-5) in two progressive steps.

4. Harvesting and counting: After washing, the intact root was excised, blot-dried and weighed. The root was then transferred to a plastic vial with 5ml scintillation cocktail (OptiPhase HiSafe3, PerkinElmer, Wellesley, USA) to be counted in a liquid scintillation counter (Beckman Instruments, Fullerton, CA, USA).

2.2.4.3 Solutions and application time

Solutions for pre-treatment and labelling were based either on MNS (Tables 2-1 and 2-2) or on MNS (-Ca), in which $Ca(NO_3)_2$ was omitted. The labelling solution was labelled with 0.01 to 0.05 mCi/l of ^{22}Na (SKS1-100UCI, Amersham Biosciences, General Electric Company, USA). Composition of pretreatment /labelling solutions and labelling times are listed in Table 2-4.

Several different rinse solutions were tested. Two solutions were selected for most of the flux experiments. The composition of the rinse solutions, rinse time and to which the experiments they were applied are listed in Table 2-5.

2.2.4.4 ^{22}Na efflux

The plants were loaded with ^{22}Na in radioactive labelled loading solution overnight. The composition of the labelling solution was the same as that used in influx experiments. The plants were illuminated and gently agitated during loading. Before being transferred to efflux solutions, the intact root of a single plant was excised and quickly dipped in ice-cold deionised water for a few second to rinse off the surface ^{22}Na. The unlabelled efflux solution was the same as the pretreatment solution for influx experiment. The root was progressively transferred to a row of 5 ml -aliquots of efflux solution at 22 time points between 0.5 and 250 min. At the end, the root was blotted dry and weighed. Each aliquot of efflux solution and the root were transferred to a plastic vial with 10 ml

scintillation solution and counted. Three replicate plants of each species were measured separately.

2.2.4.5 Data analysis and presentation

In each individual experiment the radioactivity of a 20 µl aliquot of labelling solution was counted to calculate the specific activity (the radioactivity per µmol total Na). The calculation is based on the assumption that plants do not discriminate ^{22}Na from nonradioactive Na, and the ^{22}Na flux kinetics are only affected by changes in the relative amounts of radioactive tracer in the external medium and the plant. During the early time points of the flux experiments these changes are negligible and therefore uptake and loss of ^{22}Na are directly proportional to steady-state unidirectional Na fluxes between the external medium and the root cell cytoplasm. In the influx experiments once ^{22}Na builds up in the root cells and efflux of ^{22}Na becomes apparent, the proportion of ^{22}Na in the cells that is subject to efflux changes constantly therefore the proportion of ^{22}Na in the root cells is no longer the same as in the external medium. The efflux experiments assume before efflux the root ^{22}Na was equilibrated with the labelling solution and total Na efflux can be determined from the amount of ^{22}Na released into the medium. In fact during the progress of Na efflux the proportion of ^{22}Na in the cells decreases steadily, therefore it is only appropriate to analysis the changes in the amount of ^{22}Na but not total Na in these experiments. This is why the transform of apparent rate constant is necessary.

The time course of unidirectional Na influx into the roots of Thellungiella and Arabidopsis plants is presented by plotting total Na in µmol Na/ g FW against influx time. The time course of Na uptake into the roots of Thellungiella was fitted with a two component exponential model. The initial linear part of the time courses (first 3 minutes)

were fitted with linear regressions to determine the unidirectional Na influx, and compared between Arabidopsis and Thellungiella.

Dependence of Na influx on external Na concentration in the two species is presented by plotting the rate of Na uptake into the roots of Arabidopsis after 2 min labelling, and in the roots of Thellungiella after 18 min labelling against Na concentration in the labelling solution. These curves were fitted with Michaelis-Menten models. K_m and V_{max} were extracted and compared between Arabidopsis and Thellungiella.

The effect of external Ca concentration is presented by plotting the total Na in the roots of Thellungiella after 15 min labelling against the Ca activities in the labelling solutions. This dose-response curve was fitted with a Michealis-Menten model. An inhibition curve was drawn by plotting the percentage of maximal inhibition of the Na influx against Ca activities.

The effect of channel blockers and pH are presented in a table listing the relative rates of Na uptake in percent of control (% control), determined from Na concentrations after labelling for 0.5 and 3 min in labelling solutions with 5 mM CsCl, 20 mM TEACl or 5 mM MES (pH4.1).

The kinetics of Na efflux from the roots of Arabidopsis and Thellungiella were determined by plotting the remaining Na in the roots against time. The efflux curves were fitted with the sum of three exponentials. Time constants of the individual compartments were extracted and compared between Arabidopsis and Thellungiella. To decrease the complexity of the efflux kinetics, apparent rate constants were calculated according to (MacRobbie, 1981a). In this analysis, the amount of ^{22}Na effluxed in a given time interval is expressed as a proportion of the average tissue content during the interval.

2.3 Results

2.3.1 Na concentrations after short term salt treatment

Arabidopsis and Thellungiella plants accumulate and allocate Na differently in low salt growth conditions (Figure 2-1). When grown in MNS, Thellungiella plants accumulate Na in the shoots, and keep Na concentration in the roots at a low level. Na accounted for almost 0.8% of shoot DW in control Thellungiella plants, which is about twice the amount of Na in Arabidopsis shoots (Figure 2-1). In contrast, Na made up less than 0.1% of the DW in the roots of control Thellungiella plants, which is less than a half of the Na accumulated in control Arabidopsis roots (Figure 2-1 A, B).

After 25 h treatment with 100 mM NaCl, an increase of Na concentration in the shoots was detected in both Arabidopsis and Thellungiella plants. The absolute concentrations of Na in proportion to DW in the shoots were similar between the two species after 25 h salt treatment (Figure 2-1A). However due to the higher Na concentration in the shoots of control Thellungiella plants, the Na concentration increased only by 70% in shoots of Thellungiella after salt treatment. In contrast, shoot Na concentration increased by 360% in Arabidopsis plants after 25 h salt treatment (Figure 2-1C). Na concentrations in the roots also increased substantially after salt treatment in both species. Table 2-6 lists all the p values from t tests comparing Na concentrations in shoots and roots of Arabidopsis and Thellungiella plants after salt treatments. The absolute concentration of Na in %DW after 25 h salt treatment in Thellungiella was significantly less than that in Arabidopsis ($p=0.0314$, Table 2-6). The relative increase of Na concentration after 25 h salt treatment in the roots was 820% and 930% in plants of Arabidopsis and Thellungiella respectively (Figure 2-1 D).

The shoot/root ratio of Na concentrations was with a value of 1.6/1 much lower in Arabidopsis grown in control medium than in Thellungiella, and decreased to 0.8/1 after

25 h salt treatment (Figure 2-3). In control conditions this ratio was 7.5/1 in Thellungiella, but decreased dramatically to about 1.2/1 after salt treatment (Figure 2-3). Maintenance of a high K/Na ratio has been suggested to be crucial for plant salt tolerance. It seemed that in non-saline conditions both species maintain very high K/Na ratios especially in the roots. K/Na ratio in the roots of Arabidopsis was about 37/1, and about 35/1 in Thellungiella (Figure 2-4B). In the shoots of plants grown without salt, the K/Na ratio was 11/1 in Arabidopsis, and 5.5/1 in Thellungiella (Figure 2-4A). After salt treatment, K/Na ratios in both roots and shoots of Arabidopsis and Thellungiella decreased significantly. Arabidopsis maintained K/Na ratios at 2/1 in the shoots and 2.5/1 in the roots. K/Na ratios were slightly higher in Thellungiella after 25 h salt treatment than in Arabidopsis, namely 3/1 in the shoots and 4/1 in the roots (Figure 2-4 A, B).

2.3.2 Na concentrations after long term salt stress

The difference in Na concentrations in the shoots of control plants between Arabidopsis and Thellungiella persisted over 6 weeks treatment. In both shoots and roots, Na concentration increased dramatically in both species after long-term salt treatment. Although Thellungiella plants were subjected to two times higher external salt concentrations than Arabidopsis plants (100 vs 50 mM), Na concentrations in Thellungiella were lower than in Arabidopsis (Figure 2-2A). When expressed as relative changes, after 6 weeks in 50 mM NaCl, Na concentration in the shoots of Arabidopsis was 15 times higher than the control level (Figure 2-2C). The concentration of Na in Thellungiella shoots increased only 5-fold after 6 weeks in 100 mM NaCl. This difference in the relative increase in shoot Na concentration between the shoots of Arabidopsis and Thellungiella was significant according to the t test ($p=0.0114$, Table 2-6).

Na concentration in the roots of Thellungiella was slightly higher than that of Arabidopsis after long-term salt treatment. But the increase of Na concentration in the roots relative to control level was similar between Arabidopsis and Thellungiella. Na concentration in the roots of Arabidopsis plants increased about 15-fold after long-term salt treatment (Figure 2-2B), while the increase in Thellungiella plants was about 12-fold (Figure 2-2D).

The shoot/root ratio of Na concentration in Arabidopsis plants did not change after long-term salt stress. It was around 2/1 in both control and salt treated Arabidopsis plants (Figure 2-3B). Although shoot/root ratio of Na concentration in control plants of Thellungiella was similar to Arabidopsis, it decreased to about 0.8/1 after long term salt treatment (Figure 2-3B), partly due to lower Na accumulation in the shoots than in the roots. This change in Na shoot/root allocation in Thellungiella after salt stress indicates that there is a critical barrier that limits Na translocation from roots to shoots.

K/Na ratios in the shoots of 10 weeks old control plants did not differ significantly between Arabidopsis and Thellungiella. Shoots of control Arabidopsis plants maintained a K/Na ratio of 6/1, compared to 4/1 in control Thellungiella plants (Figure 2-4C). K/Na ratios in the roots were higher than in the shoots. Roots of control Arabidopsis plants maintained a K/Na ratio of 23/1 (Figure 2-4D). This ratio in the roots of control Thellungiella plants was about half of the Arabidopsis ratio (12.5/1, Figure 2-4D). After long-term salt stress, K/Na ratios decreased significantly in the roots and shoots of both species. After salt treatment, the K/Na ratio was 0.3/1 in the shoots of Arabidopsis plants, and 0.67/1 in Thellungiella (Figure 2-4C). In the roots the K/Na ratio was 1.25/1 in Arabidopsis, and 1.1/1 in Thellungiella (Figure 2-4D). Although absolute values of K/Na ratios were lower in Thellungiella than those in Arabidopsis,

the relative decrease of K/Na ratio during salt stress, especially in the roots, was smaller in Thellungiella than in Arabidopsis.

2.3.3 Time course of net Na uptake

The time course of Na accumulation in roots and shoots of Arabidpsis and Thellungiella plants was recorded over 72 h treatment at 100 mM NaCl. The kinetics of net Na uptake in the shoots of Arabidopsis and Thellungiella can be fitted with single exponentials (Figure 2-5A). The Na concentration in shoots of control Thellungiella plants was about 5.2 mg/g shoot DW (Table 2-10, t=0). The maximal shoot Na concentration in Thellungiella plants was about 10 mg/g shoot DW (Table 2-10, t=∞), with a time constant of 17.2 h. In shoots of Arabidopsis plants, the Na concentration was 2.8 mg/g shoot DW before salt stress, with a maximum increase to 28.7 mg/g shoot DW after salt stress (Table 2-10), and a time constant of 66.7 h (Table 2-10). The maximal net Na uptake in the shoots of Arabidopsis was about 3 times of that in Thellungiella (Figure 2-5A).

The average rate of net Na uptake into the shoots of Thellungiella over the first 6 h of salt treatment was about 0.021 μmol/g shoot DW/min. After 24 h, the rate of net Na uptake into the shoots of Thellungiella has decreased to about 0.0023 μmol/g shoot DW/min. The initial rate of net Na uptake in shoots of Arabidopsis plants over 6 h was approximately 0.039 μmol/g shoot DW/min. It decreased to 0.015 μmol/g shoot DW/min after 24 h salt treatment.

The kinetics of net Na uptake into the roots of Arabidopsis and Thellungiella were fitted with double exponentials with a constant offset (Figure 2-5B, Table 2-10). Over the initial 6 h of salt treatment Na was taken up into the roots at approximately similar rates by Arabidopsis and Thellungiella. After 6 h in 100 mM NaCl, net Na uptake into the roots of Thellungiella plants almost ceased, whereas in Arabidopsis plants, root Na

concentration still increased steadily. Initial rates of net Na uptake into the roots over the first 6 h were 0.064 μmol/g root DW/min in Thellungiella and 0.048 μmol/g root DW/min in Arabbidopsis. After 24 h salt treatment, this rate dropped to 0.0004 nmol/g root DW/min in Thellungiella, and 0.003 nmol/g root DW/min in Arabidopsis.

The differences in tissue Na concentrations determined after 24 h in this experiment are in good agreement with those determined in the 25 h experiment (compare Figure 2-5 with Figure 2-1). The experiment shows that similar shoot Na concentrations in the two species after the 25 h salt treatment were of transient nature.

2.3.4 Apoplastic binding of Na in Thellungiella

Prior to carrying out ^{22}Na flux experiments I tested a rage of washing procedures for their suitability to remove apoplastically bound ^{22}Na.

2.3.4.1 Apoplastic washing kinetics

To establish the kinetics of apoplastic Na removal, excised roots of Thellungiella were killed by boiling in water for 10 min, then labelling with ^{22}Na for 10 min in loading solution with 100 mM NaCl (Table 2-4). In Figure 2-7 the residual apoplastic Na in boiled roots is plotted against rinse time. In a standard rinse solution with 10 mM $CaCl_2$ and 100 mM NaCl, it took 3 min to wash off most of apoplastic bound Na from the boiled roots. Longer washing time, e.g. 5 min, did not significantly improve washing efficiency.

2.3.4.2 High residual Na binding

The level of residual apoplastic Na after washing was higher in Thellungiella than in Arabidopsis (Tables 2-8, 9). Apoplastic binding of Na was tested in boiled roots of Arabidopsis with the standard rinse solution and a rinse solution with 2 mM La (+La) (Table 2-9). The residual Na binding of Arabidopsis was at least 2 to 3 fold lower than

that of Thellungiella, although the Arabidopsis binding levels were so low that the counts did not accurately represent the values of the residual binding (Table 2-9). This would normally over-estimate the concentration, which supports that apoplastic Na binding was lower in Arabidopsis root.

2.3.4.3 Trivalent cations in the washing solution

Additional trivalent cations in the rinse solution, e.g. 2 mM La or 5 ml Gd, and low pH (pH4) had no obvious effect in improving washing efficiency. The levels of residual apoplastic Na after washing in the different rinse solutions for 10 min were similar to the levels after washing for 3 min (Table 2-8).

2.3.4.4 Washing without Na

Considering that washing with a high concentration of Na will prevent exchange of apoplastically bound Na with other cations, a rinse solution without NaCl was tested to improve washing efficiency. Surprisingly washing solely with 75 mM $CaCl_2$ or 100 mM KCl, did not succeed in exchanging all the apoplastically bound Na from the boiled roots of Thellungiella (Table 2-8).

2.3.4.5 Dependence of apoplastic binding of Na on the external Na concentration and labelling time

Apoplastic Na binding in boiled roots of Thellungiella increased with labelling time for 0.9 μmol Na/g FW Root/min during the first 10 min of labelling. The increase slowed down after 10 min of labelling (Figure 2-6). Apoplastic Na binding in the boiled roots increased with the Na concentration in the labelling solution in both Arabidoposis and Thellungiella (Figure 2-9). The residual Na binding of Thellungiella after 3 min washing in the rinse solution with 2 mM La was always approximately twice as high as that of Arabidopsis.

2.3.4.6 Ca concentration and channel blockers had no effect on Thellungiella root apoplastic binding

Ca concentration in the labelling solution did not affect the apoplastic Na binding in boiled roots of Thellungiella. Boiled roots of Thellungiella were labelled in labelling solutions with 100 mM NaCl and 0.1, 0.5 or 10 mM Ca for 10 min then washed for 3 min to compare the effect of Ca concentration on apoplastic Na binding. There was no significant difference between residual apoplastic Na in boiled roots labelled in different Ca concentrations (Figure 2-8). Boiled roots of Thellungiella and Arabidopsis were pre-treated for 3 min in unlabelled loading solution with additional blockers or pH buffer before labelling for 0.5 and 3 min in ^{22}Na loading solutions to calculate the rate of Na apoplastic binding. It seemed that additions of various blockers in the labelling solution, e.g. 5 mM CsCl, 20 mM TEA-Cl and pH4.1 in 5 mM MES, even increased the rate of apoplastic Na binding in boiled root of Thellungiella (data not shown).

2.3.5 Unidirectional Na influx into the roots

Steady-state unidirectional Na influx was determined by measuring accumulation of external ^{22}Na in the roots. A 60 min time course of ^{22}Na influx into the roots of Thellungiella plants was recorded in ^{22}Na labelled medium with 100 mM NaCl and 0.1 mM $CaCl_2$. The shape of this time course reflects a typical influx curve with an initial linear rise and subsequent attenuation due to increasing ^{22}Na efflux from the root cells back to the external medium (Figure 2-10A). The first 3 min of ^{22}Na uptake, assumed to reflect steady-state unidirectional influx of Na, were compared between Thellungiella and Arabidopsis (Figure 2-10B). Linear regression fits of these data revealed rates of 0.66 ± 0.12 and 0.31 ± 0.02 μmol Na /g root FW/min for unidirectional Na influx into roots of Arabidopsis and Thellungiella respectively (Table 2-10). Thus unidirectional

Na influx into the roots is more than two times faster in Arabidopsis than in Thellungiella.

2.3.6 Na influx into the shoots

^{22}Na was detected in the shoots of Thellungiella after 30 min labelling (Figure 2-11). However it is difficult to determine the exact amount of ^{22}Na in the roots so as to calculate the Na influx into the shoots from the roots (see discussion). Nevertheless, the time courses of Na influx into the roots and shoots over a period of 24 h in ^{22}Na labelled 100 mM NaCl were calculated based on the assumption of no Na efflux from the roots or shoot-root Na re-circulation. The calculated initial unidirectional Na influx into the shoots is 0.34 μmol/g FW shoot/ h, but the real value should be higher than this. The steady-state shoot/ root ratio of Na concentration increased from 1/12.4 at 30 min to 1/5.3 at 1 h and 1/3.2 at 24 h after salt application. Unfortunately the corresponding data for Arabidopsis were not collected.

2.3.7 Dependence of Na influx on external Na concentration

Na dependence of the rate of Na influx into roots of Arabdipsis and Thellungiella was determined by measuring ^{22}Na levels after 2 min and 18 min of uptake respectively (for usage of different time points see Discussion) in ^{22}Na labelled growth solutions with 3 mM Ca activity and increasing Na concentrations. Again all plants were pre-treated for a week in the MNS with respective Na concentrations to achieve steady state. ^{22}Na influx into the roots of Thellungiella increased gradually with external Na concentration up to 400 mM NaCl with no saturation. ^{22}Na uptake into the roots of Arabidopsis also increased with external Na but the increase was much steeper than in Thellungiella. Na concentrations above 100 mM could not be applied to Arabidopsis because they led to plant death. Figure 2-12 presents dose-response curves plotting Na influx against external Na concentration. Both curves were tentatively fitted with Michaelis-Menten

models of K_m values of 102 mM and 671 mM and V_{max} values of 0.79 and 0.66 μmol/g root FW/min for Arabidopsis and Thellungiella respectively (Table 2-10). However, for Arabidopsis these values are not reliable due to the lack of data at high Na concentrations (See Discussion).

2.3.8 Inhibition of Na influx by extracellular Ca

Inhibition of unidirectional Na influx by external Ca was found in Arabidopsis, wheat and maize (Essah et al., 2003; Davenport et al., 1997; Zidan et al., 1991). Patch clamp studies with root protoplasts from these species indicated that the inhibition is partly due to Ca inhibition of Na permeable voltage-independent channels (Demidchik and Tester, 2002; Roberts and Tester, 1997a; Tyerman et al., 1997). The effect of external Ca on Na influx into the roots of Thellungiella was determined by measuring ^{22}Na levels in the roots after 15 min incubation in ^{22}Na labelled medium with 100 mM NaCl and various Ca activities. Similar to other species Na influx into the roots of Thellungiella was strongly but not completely inhibited by external Ca (Figure 2-13). The plot of Na influx against external Ca concentration revealed a K_i of 160 ± 30 μM (Figure 2-13 and Table 2-10). However, Ca only partially inhibits Na influx with approximately 23% of the maximum Na influx remaining at 3 mM external Ca activity.

2.3.9 Na influx into roots is not inhibited by Cs and TEA

Cs and TEA block inward- and outward- rectifying voltage-dependent K channels respectively (Very and Sentenac, 2003). Voltage-independent cation channels are not affected by these blockers (Maathuis and Sanders, 2001; Demidchik and Tester, 2002; Volkov and Amtmann, unpublished results). To assess the contribution of these channel types to unidirectional Na uptake, effects of Cs and TEA on root unidirectional Na influx (the initial 5 min) were measured in the two species (Table 2-11). Influx after addition of 5 mM CsCl was 135.4 ± 2.7 % and 161.0 ± 3.6 % of control influx in

Arabidopsis and Thellungiella respectively. After addition of 20 mM TEACl influx increased to 192.1 ± 2.2 % in Arabidopsis and 156 ± 4.5 % in Thellungiella. Thus neither Cs nor TEA inhibited unidirectional Na influx into roots of Arabidopsis or Thellungiella indicating that the influx is not mediated by voltage-dependent K channels.

2.3.10 Unidirectional Na efflux from roots

To determine the kinetics of Na efflux from roots, Arabidopsis and Thellungiella plants were loaded with ^{22}Na for 24 h and excised roots were subsequently transferred to unlabelled solution containing 100 mM NaCl. The time course of ^{22}Na release into the external medium was measured over 250 min by transferring the roots into fresh solution at given time points and measuring the radioactivity in the medium samples. Assuming that in both species root ^{22}Na levels were equilibrated with the external medium at the beginning of the experiment total Na efflux can be determined from the amount of ^{22}Na released into the medium. Figure 2-14A shows the time courses of Na efflux for both species. Best fits were achieved with equations for an exponential decay described by the sum of three exponentials plus a constant offset thus reflecting the contribution of at least four different types of Na pools within the roots (Table 2-10). Quantitative analysis of the measured efflux kinetics revealed a complex arrangement of exchangeable Na pools. This was expected as the experiments were carried out with whole roots and therefore reflected the transport across different cellular compartments and tissues. Due to this complex arrangements of Na pools, assignment of the kinetic components to individual compartments is difficult. The first very fast component is likely to represent loosely bound apoplastic Na. The second component with time constants of 2.3 ± 0.33 and 0.61 ± 0.08 min in Arabidopsis and Thellungiella respectively most likely combines apoplastic and cytoplasmic efflux components from various tissues. Finally, the third component with time constants of 37.5 ± 4.2 and 42.41

± 4.10 min respectively is thought to consist of cytoplasmic and vacuolar contributions from various tissues. A pool of Na that did not exchange with the external medium over the assessed period of time, apparent as a constant offset in the fitted equation, might reflect Na trapped in the root xylem.

To transform the efflux kinetics into a more comparable format, apparent rate constants were calculated according to MacRobbie (1981b). This analysis takes into account the amount of ^{22}Na remaining in the tissue at any point of the efflux time course. Thus ^{22}Na release over a given period of time (i.e. between two adjacent time points) is related to the mean ^{22}Na concentration present in the tissue over this period of time. A plot of apparent efflux rate constants against time is shown in Figure 2-14B. The resulting curve shows that apart from the first minute of the experiment efflux rate constants were always higher (i.e. efflux was faster) in Arabidopsis than in Thellungiella.

2.4 Discussion

2.4.1 Na apoplastic binding in the roots of Thellungiella

A high apoplastic Na binding specificity was found in boiled roots of Thellungiella. The residual Na bound to the boiled root material after washing was unexpectedly high and could not be exchanged by Ca or K ions. Binding capacity of the cell wall for cations is mainly due to negatively charged pectins, but in this interaction monovalent cations can usually be easily replaced by bivalent or trivalent cations. The findings of this study raise the possibility that there are other proteins in the cell walls of Thellungiella roots that selectively bind Na. However one also has to consider that destruction of root structures by boiling might expose more ion binding sites than present in living roots, and that this difference could be higher in Thellungiella than in Arabidopsis. In fact, influx and efflux experiments carried out with intact tissue did not indicate higher apoplastic binding in Thellungiella (e.g. time point 0 in Figure 2-10B). Initial Na efflux

from intact roots was even faster in Thellungiella than in Arabidopsis (Figure 2-14B). Apoplastic binding capacity for Na has important implications for osmotic adjustment of root cells to high salinity. A detailed biochemical analysis of cell wall components is required to identify differences between Arabidopsis and Thellungiella and assess their effect on water relations.

2.4.2 Accumulation and allocation of Na in low salt conditions

Na shoot/root ratios in Arabidopsis were fairly stable with values around 1-1.5 in younger plants and 2 in older plants, and did not show dramatic changes during salt treatment. By contrast, young Thellungiella plants accumulated much more Na in the shoots than in the roots (shoot/root ratio $\approx$ 7) in low salt conditions. This difference did not exist after salt treatment and was also not present in older plants (Figure 2-3). Preferential allocation of Na in the shoots of young Thellungiella plants in low salt was accompanied by higher total amount of Na in these plants than in Arabidopsis. It is likely that in Thellungiella Na plays an important role as an osmoticum for growth under low salt conditions. There are several possible reasons for the difference in Na accumulation between Thellungiella and Arabidopsis during salt treatment. Either the transport pathway which supplies Na to Thellungiella in low salt conditions is quickly down-regulated, or this transport system is saturated in low mM NaCl ('high affinity uptake'). The observation that preferential shoot allocation disappears during salt treatment suggests that a root – shoot barrier, e.g. at the xylem parenchyma, is established under salt stress. Alternatively, Na might be recycled from the shoot to the root. Identification of the physiological and molecular nature of Na tissue allocation requires analysis of the kinetics and pharmacology of ^{22}Na uptake into the shoots as well as electrophysiological characterisation of Na currents in the relevant tissues. My

result of ^{22}Na uptake into the shoots (section 2.3.6) was only preliminary therefore no data are available in either aspect so far.

2.4.3 Thellungiella in saline conditions; salt-accumulator, salt-extruder or salt excluder?

A significant difference in Na accumulation between Arabidopsis and Thellungiella can be detected within a few hours after addition of salt to the medium. The relative increase of the shoot Na concentration after salt treatment for 25 h in Thellungiella was half of the relative increase of Na in the shoots of Arabidopsis (Figure 2-1A, C). Net Na uptake continued to be considerably lower in Thellungiella than Arabidopsis over a time course of 3 days (Figure 2-5). After salt treatment for 6 weeks the shoots of Thellungiella accumulated Na equivalent to about 3% shoot DW which equals to half the amount of Na in the shoots of Arabidopsis plants grown in only half of the salt concentration for the same period of time (Figure 2-2A, C). Clearly, Thellungiella does not over-accumulate Na to adapt to high external salinity.

However, Thellungiella is not a salt-extruder either. Lower Na accumulation in Thellungiella is not due to elevated active Na efflux from the roots. Rather Thellungiella effectively excludes Na from roots. The unidirectional Na influx into the roots of Thellungiella is 0.31 μmol/g root FW/min, less than half the rate in Arabidopsis which is 0.66 μmol/g root FW/min (Table 2-10). With such a low Na influx it is not necessary for Thellungiella to have higher export rate than Arabidopsis. This is evident when comparing the measured rates of unidirectional influx with those of net Na uptake into the plants (Table 2-12). Taking into account an average shoot/root dry matter ratios of 7/1 in Arabidopsis and 3.5/1 in Thellungiella the measured difference of root and shoot Na concentrations between control and salt treated plants (Figure 2-1) is equivalent to a whole plant net Na uptake of 102 mg Na/g root DW in Arabidopsis and 29.3 mg Na/g

root DW in Thellungiella over 25 hours. Considering that FW/DW ratios were 20 and 12 in roots of Arabidopsis and Thellungiella respectively the measured unidirectional Na influx into the roots of 0.66 and 0.31 μmol Na/g root FW/min would lead to a net uptake of 455.4 and 128.3 mg Na/g root DW over 25 hours in Arabidopsis and Thellungiella respectively, if not counteracted by Na efflux. This suggests not only that in both species a large and similar proportion (77-78%) of the Na taken up into the plant is exported back to the external medium, but also that absolute amounts of Na efflux are smaller in Thellungiella than in Arabidopsis. Direct measurements of Na efflux from the roots of both species confirmed this result (see 2.4.4).

2.4.4 Identification of Na uptake pathways

^{22}Na influx into the roots of Thellungiella showed a typical time course similar to the one measured in Arabidopsis (Essah et al., 2003). Na influx into the roots of Arabidopsis has already been characterized in great detail with ^{22}Na flux technique and patch clamping (Demidchik and Tester, 2002; Essah et al., 2003). Non-selective voltage independent channels are considered as the most likely pathway responsible for Na influx into roots of Arabidopsis. Na current through this channel type is inhibited by external Ca, but not by K channel blockers, e.g. Cs and TEA (Demidchik and Tester, 2002) and the same is true for ^{22}Na influx in Arabidopsis (Essah et al., 2003).

The root unidirectional Na influx increased with external Na concentration in both Arabidopsis and Thellungiella. However different influx time was used to calculate the Na influx for the two species (2 min for Arabidopsis and 18 min for Thellungiella). The longer influx time used for Thellungiella led to lower Na influx, e.g. root Na at 2 min was about a quarter of the root Na at 18 min after salt treatment, therefore the rate of Na influx after 18 min salt treatment would be about half of the rate after 2 min. Although the use of different influx times under-estimated the rate of unidirectional Na influx for

Thellungiella, the real values would still be smaller than those of Arabidopsis, and the shape of the Na dependent curve probably would not be affected. Na-dependence of Na influx was tentatively fitted although saturation had not yet been reached at the maximal Na concentrations applied to both species. The fitted K_m values were in the range of several hundred mM indicating that even if saturable the transporters mediating Na influx had very low affinity (especially in Thellungiella, K_m = 671 mM). It is therefore likely that in both species ion channels are the main pathway for Na influx at high salinity.

Unidirectional Na influx into the roots of Thellungiella is inhibited by external Ca (Figure 2-12) but not by Cs or TEA (Table 2-11) suggesting that voltage-independent channels are also responsible for Na uptake into the roots of Thellungiella. An unexpected enhancement of Na influx by additional Cs and TEA was found for both Arabidopsis and Thellungiella. This could be explained with a hyperpolarization of the root cells due to decreased K permeability of voltage dependent channels by application of the blockers, which in turn provides additional driving force for Na uptake through channels that are not blocked by these compounds. A more detailed analysis of the effects of Cs and TEA on membrane potential and K conductance in the two species is required to fully explain these data.

The finding that the inhibitor profile of Na influx is similar in Arabidopsis and Thellungiella suggests that it is mediated by the same type of channel in the two species. To explain the difference in the Na influx this channel type must have lower activity or Na permeability in Thellungiella than Arabidopsis. Indeed, quantitative data for conductance and ion selectivity of the voltage-independent cation channels in Thellungiella differ from Arabidopsis and other glycophytic species, which suggests that the respective channel proteins have species-specific structural features (Volkov et

al., 2004; Volkov and Amtmann, unpublished results). No genes for Na uptake channels have been identified so far in plants, although members of the cyclic-nucleotide gated channel (CNGC) gene family have emerged as likely candidates (Maathuis and Sanders, 2001). Identification of the genes underlying root Na uptake in Thellungiella and Arabidopsis is one of the major challenges for the future and will allow us to further study their role in salt tolerance. In particular structure-function analysis is likely to reveal important differences between ion channel proteins of the salt-sensitive and the salt-tolerant species with respect to their K/Na selectivity.

2.4.5 Na efflux

According to the quantitative comparison of the measured unidirectional influx with those of net Na uptake into the plants, in both Arabidopsis and Thellungiella a large proportion of the Na taken up into the plant is exported back into the external medium. Even if taking into account different Ca levels in the media used for determining net Na accumulation (0.5 mM Ca) and unidirectional Na influx (0.1 mM Ca) and their inhibitory effect on Na influx, as well as the fact that low transpiration during the dark period will reduce the average influx over the 25 h period, unidirectional Na influx is still two times higher than the net Na uptake. The difference will rise again if we consider that net Na accumulation was determined in non-steady state conditions and therefore includes an initial period of high uptake rates. Based on these assumptions, calculated absolute unidirectional efflux was between 0.44 and 0.51 μmol Na/g root FW/min for Arabidopsis and between 0.13 and 0.24 μmol Na/g root FW/min for Thellungiella roots. Efflux of this magnitude were indeed observed within the first 10 min of the efflux time course, suggesting that the cytoplasmic component is the main contributor to ^{22}Na efflux over this period of time.

Differences in Na efflux curves between Arabidopsis and Thellungiella were very small, and the variation of the data did not allow statistically significant separation (Figure 2-14A). However, the apparent efflux rate constants showed that Na efflux is generally higher in Arabidopsis than in Thellungiella (Figure 2-14B). This finding agrees with the above calculations.

In conclusion, the majority of total Na taken up from the saline medium was quickly exported back to the external solution by both species. Interestingly, the proportion of Na that is exported is similar in Arabidopsis and Thellungiella indicating that the efflux is adjusted by the unidirectional Na influx. In this sense the halophyte and the glycophyte are both 'salt-extruders'. Salt extrusion *per se* should therefore not be considered as a parameter for distinguishing between glycophytes and halophytes. However, absolute amounts of Na fluxes are higher in Arabidopsis. Arabidopsis plants therefore must spend a large amount of energy on active transport. This might be an important factor in limiting its growth in a saline environment. Nevertheless it has been shown that over-expression of the plasma membrane Na/H export system (SOS1) in Arabidopsis can increase salt tolerance (Shi et al., 2003), which indicates that Na export in wild type Arabidopsis is not operating at its energetic limit. In summary, both Arabidopsis and Thellungiella export a large proportion of the Na taken up but due to higher unidirectional influx the glycophytic species spends more energy on Na efflux, and is still not capable of reducing its net Na uptake to the low rate observed in the halophyte.

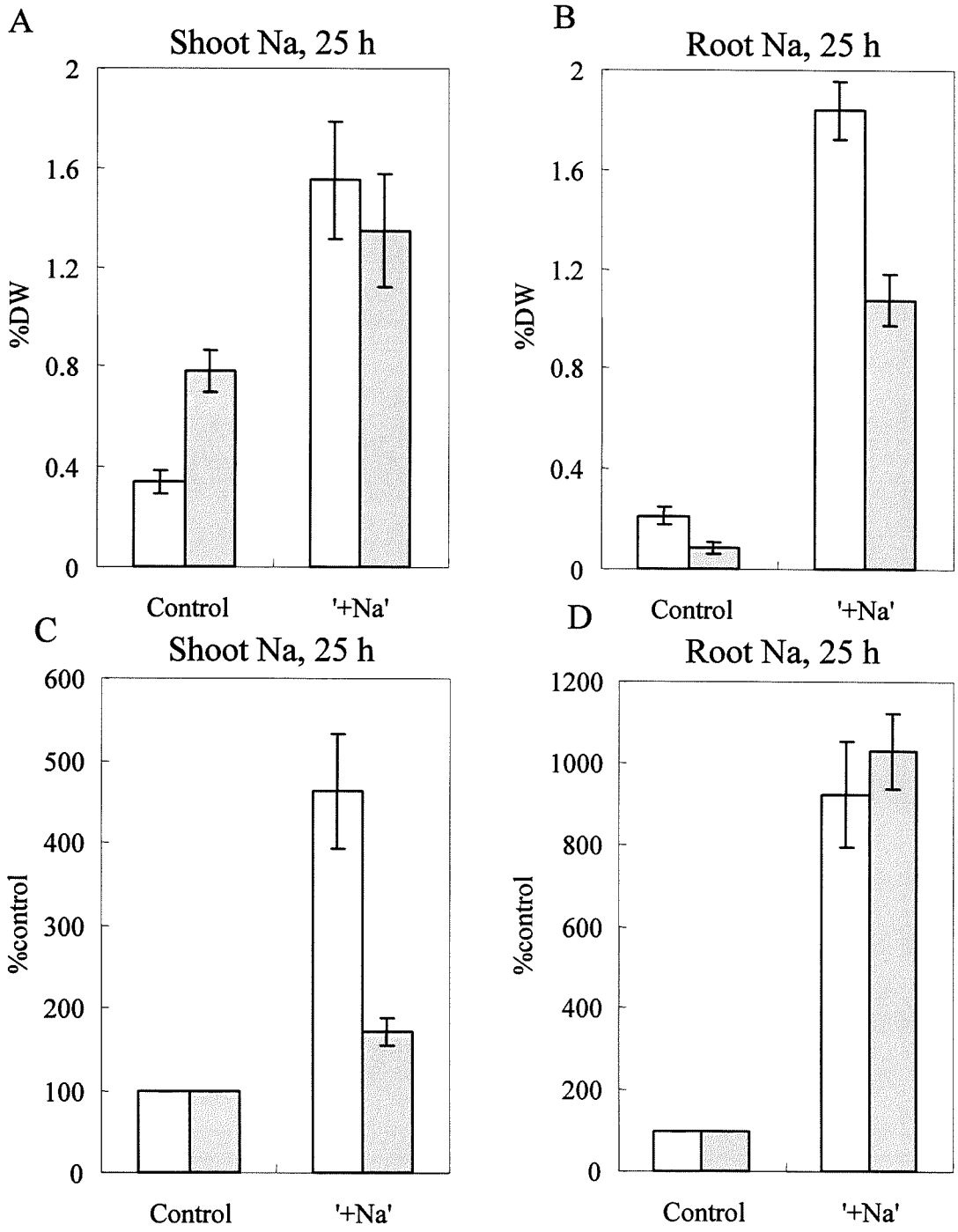

Figure 2-1. Sodium concentrations of Arabidopsis (white bars) and Thellungiella (grey bars) plants after 25 h exposure to MNS ('control') or MNS + 100 mM NaCl ('+Na'). Six to seven plants were pooled for each replicate and analysed with ICP-OES. Values are the mean (n=4) ± SE. Presented are absolute values as percent of dry weight in shoot (A) and root (B), and relative changes to the control level within each experiment in shoot (C) and root (D). For statistical analysis see Table 2-6.

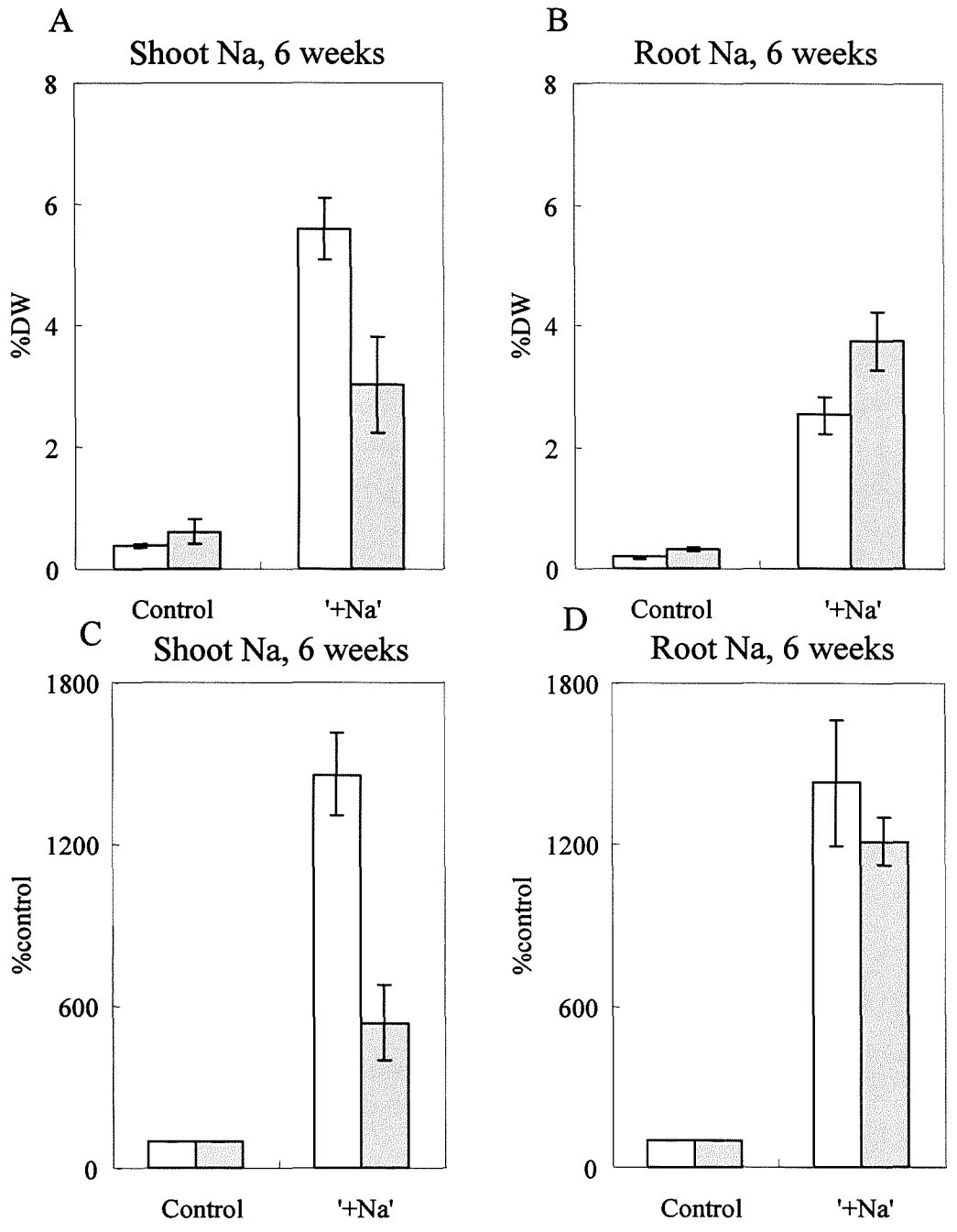

Figure 2-2. Sodium concentrations of Arabidopsis (white bars) and Thellungiella (grey bars) plants after 6 weeks exposure to 'control' or ' Na' (MNS+50 mM NaCl for Arabidopsis, MNS+100 mM NaCl for Thellungiella) medium. Six to seven plants were pooled for each replicate. Values are the mean (n=4) ± SE. Presented are absolute values as percent of dry weight in shoot (A) and root (B), and relative changes to the control level within each experiment in shoot (C) and root (D). For statistical anaysis see Table 2-6.

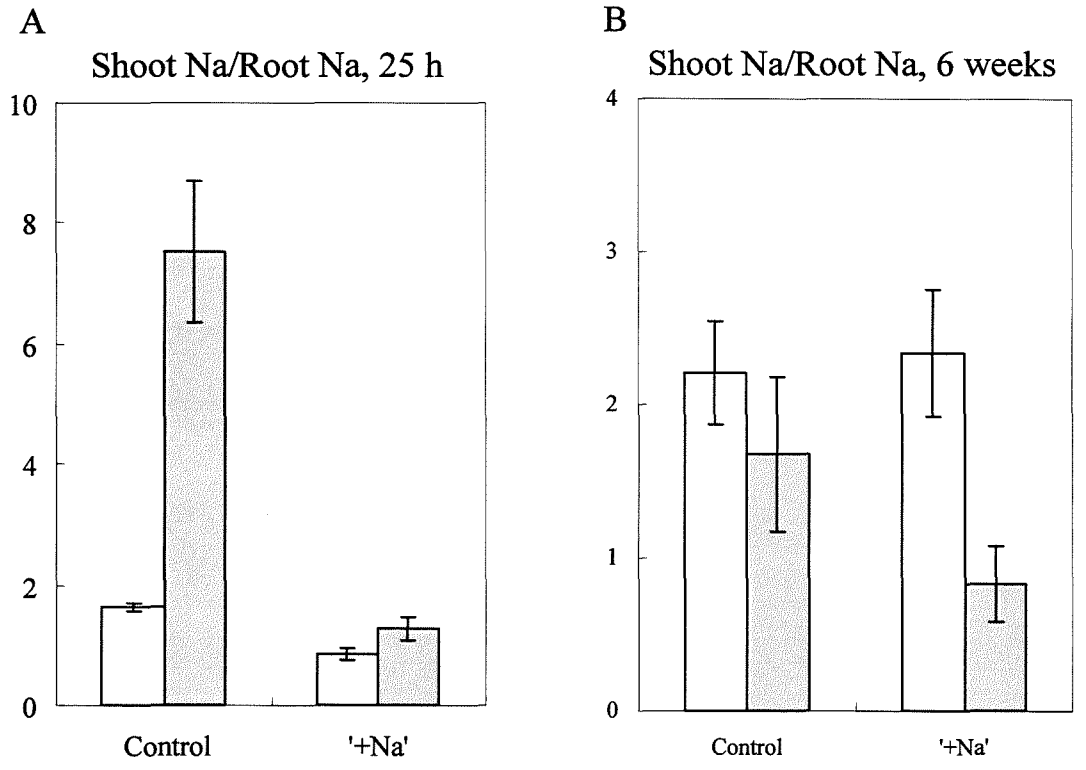

Figure 2-3. Shoot/ root ratios of Na concentration in Arabidopsis (white bars) and Thellungiella (grey bars) plants with and without short- term (A) and long-term (B) salt treatment. For the short-term salt treatment plants were exposed for 25 h to MNS with 100 mM NaCl. For the long-term salt treatments plants were exposed for 6 weeks to MNS with 50 mM NaCl (Arabidopsis) or 100 mM NaCl (Thellungiella). Six to seven plants were pooled for each replicate. Values are the mean (n=4) ± SE. For statistical analysis see Table 2-7.

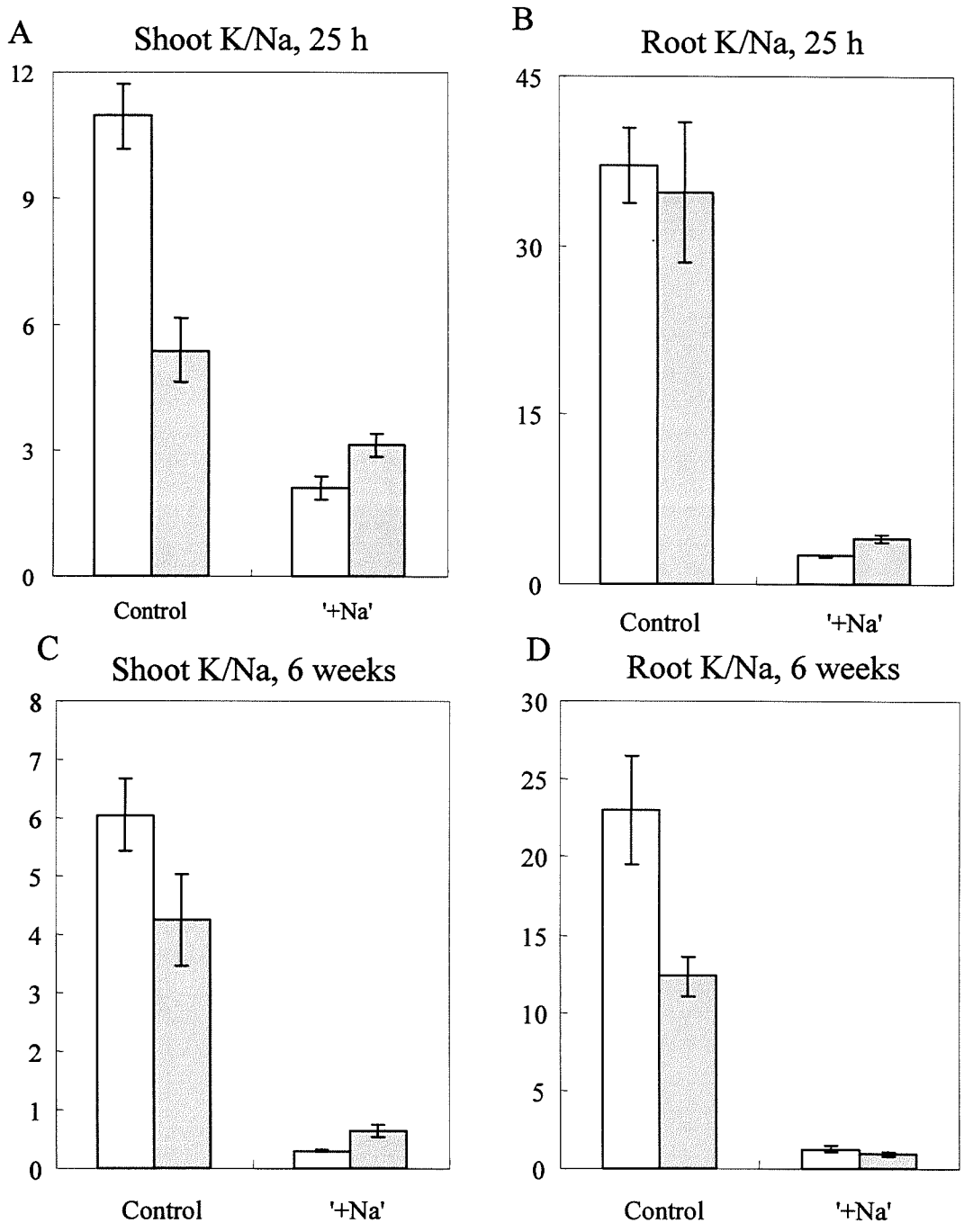

Figure 2-4. K/Na ratios in Arabidopsis (white bars) and Thellungiella (grey bars) plants with and without short-term (A and B) and long- term (C and D) salt treatment. For the short-term salt treatment plants were exposed for 25 h to MNS with 100 mM NaCl. For the long-term salt treatments plants were exposed for 6 weeks to MNS with 50 mM NaCl (Arabidopsis) or 100 mM NaCl (Thellungiella). Six to seven plants were pooled for each replicate. Values are the mean (n=4) ± SE. For statistical analysis see Table 2-6.

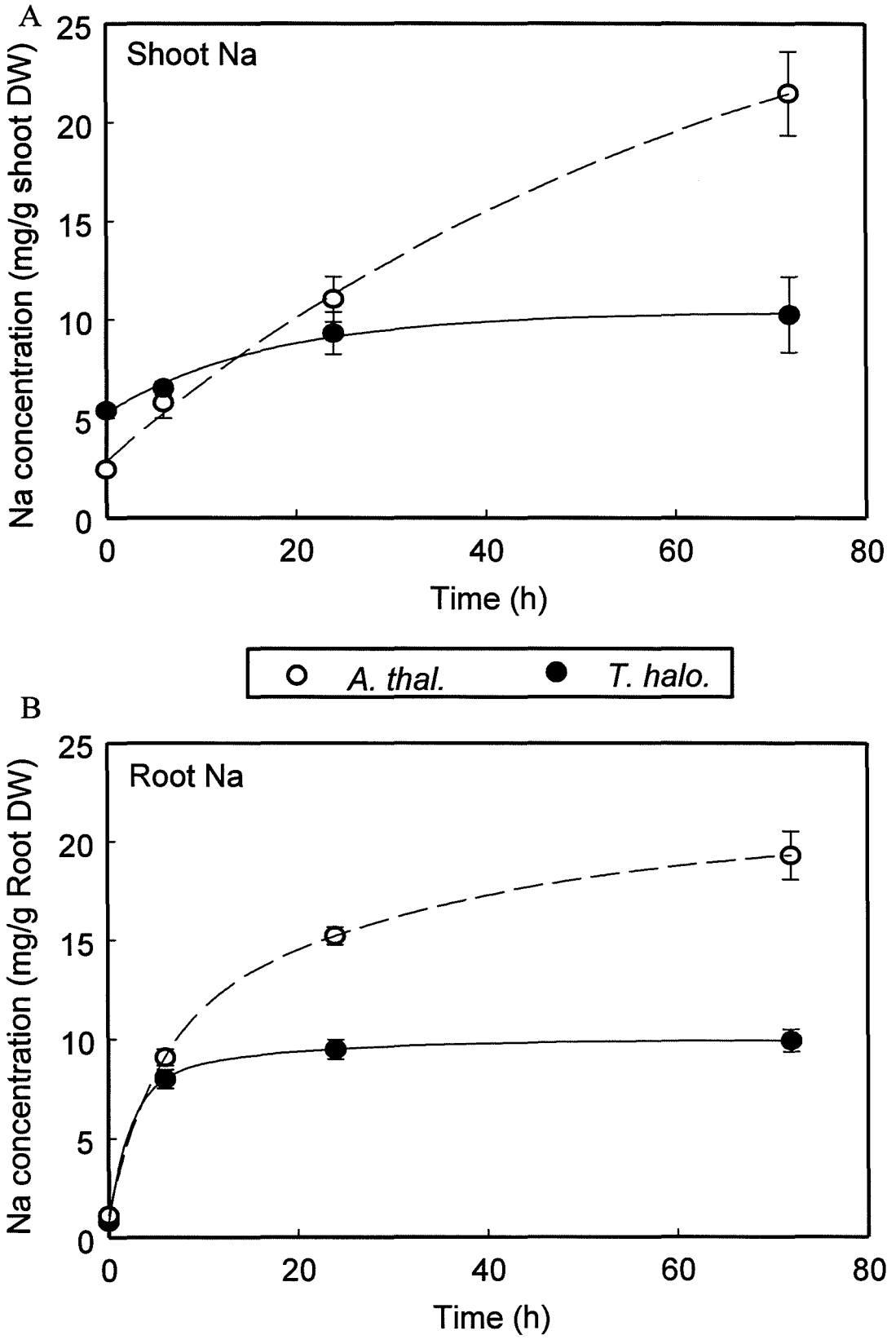

Figure 2-5. Kinetics of net accumulation of Na in shoots (A) and roots (B) of Arabidopsis (open circles) and Thellungiella (closed circles) during 72 h treatment with 100 mM NaCl in MNS. Ion concentrations of single plants were determined by ICP-OES. Values are mean ± SE (n=9).

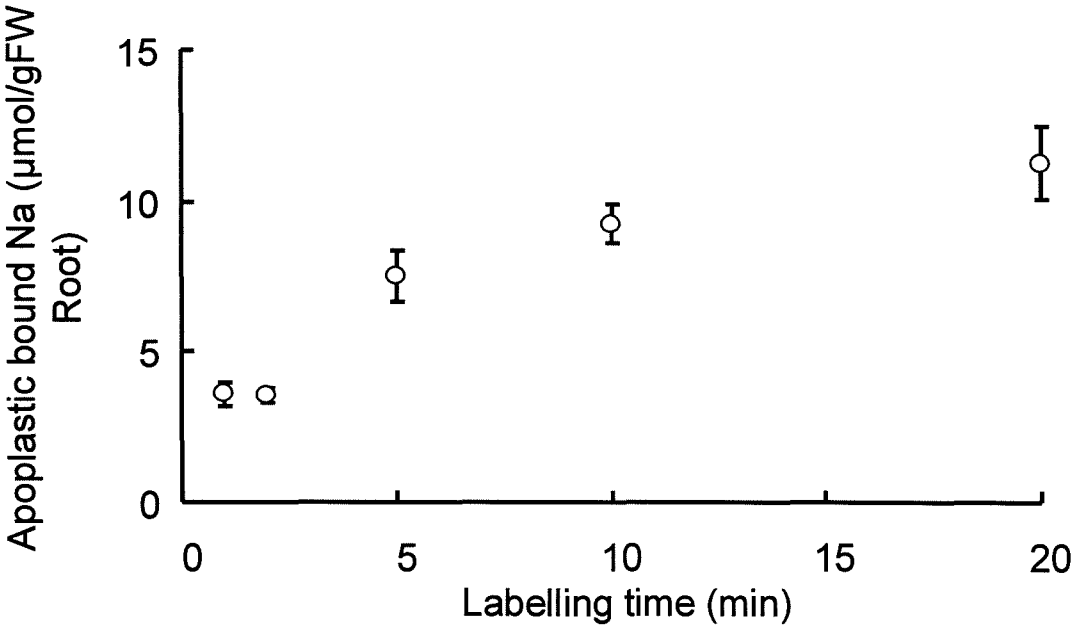

Figure 2-6. Time course of apoplastic binding of Na in boiled roots of Thellungiella in ^{22}Na labelled solution with 100 mM NaCl. Residual apoplastic Na in proportion to root fresh weight after 3 min washing is plotted against labelling time. Values are mean ± SE (n=4).

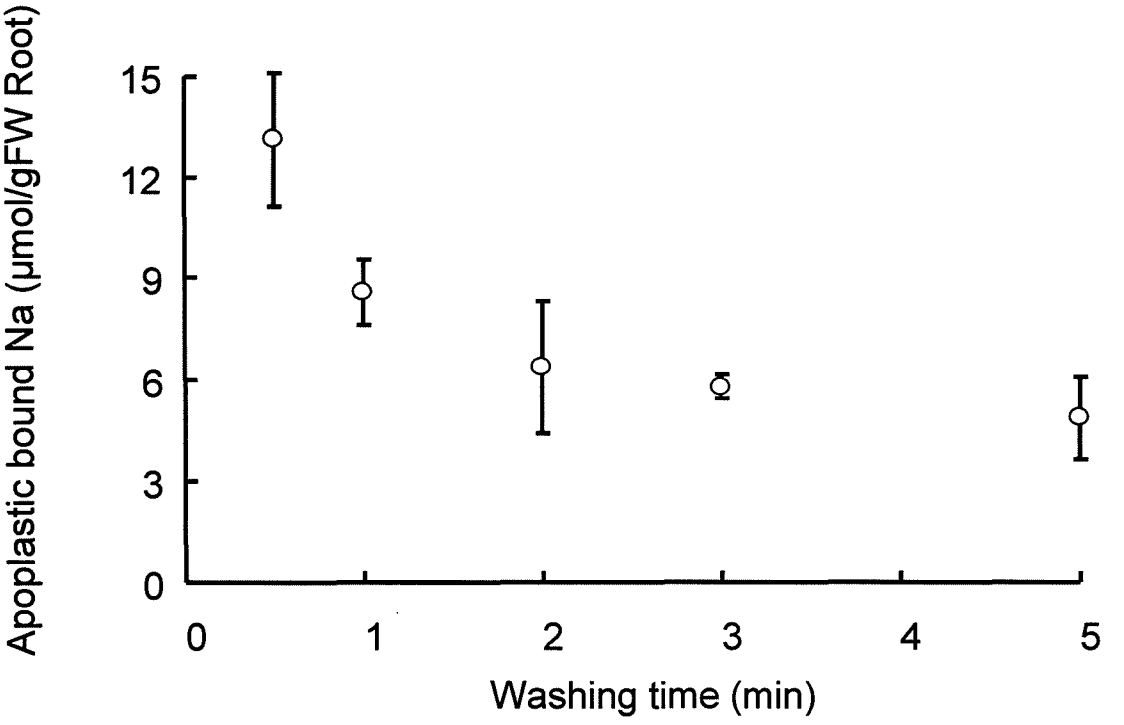

Figure 2-7. Time course of washing of boiled roots of Thellungiella after 10 min labelling in ^{22}Na labelled solution with 100 mM NaCl. The residual apoplastic Na is plotted against time of washing in an ice-cold standard washing solution with 100 mM NaCl and 10 mM $CaCl_2$. Values are mean ± SE (n=4).

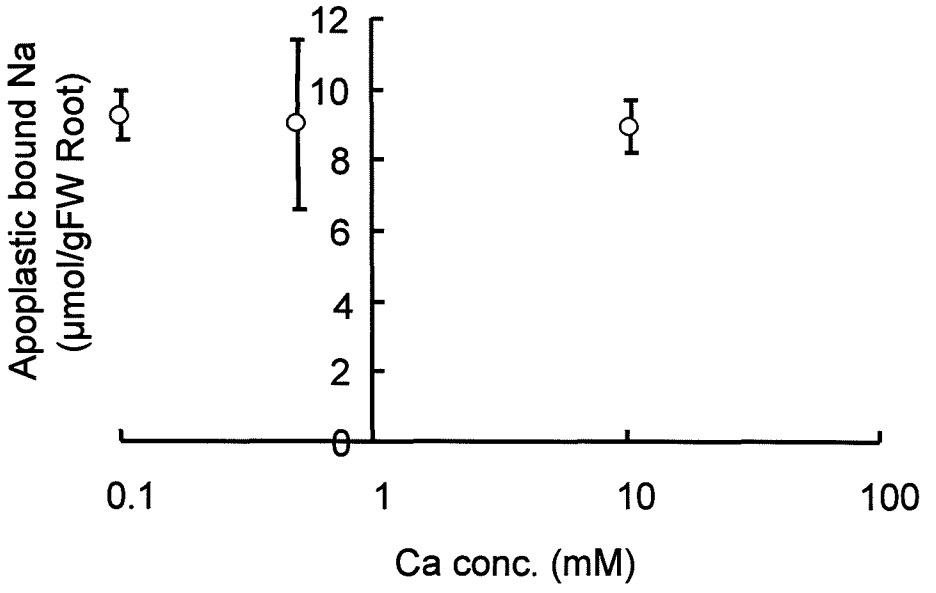

Figure 2-8. Effect of Ca concentration in the labelling solution on apoplastic Na binding in boiled roots of Thellungiella. The residual apoplastic Na after standard washing precedure is plotted against the Ca concentration (on log scale) in the ^{22}Na labelled solution with 100 mM NaCl. Values are mean ± SE (n=4).

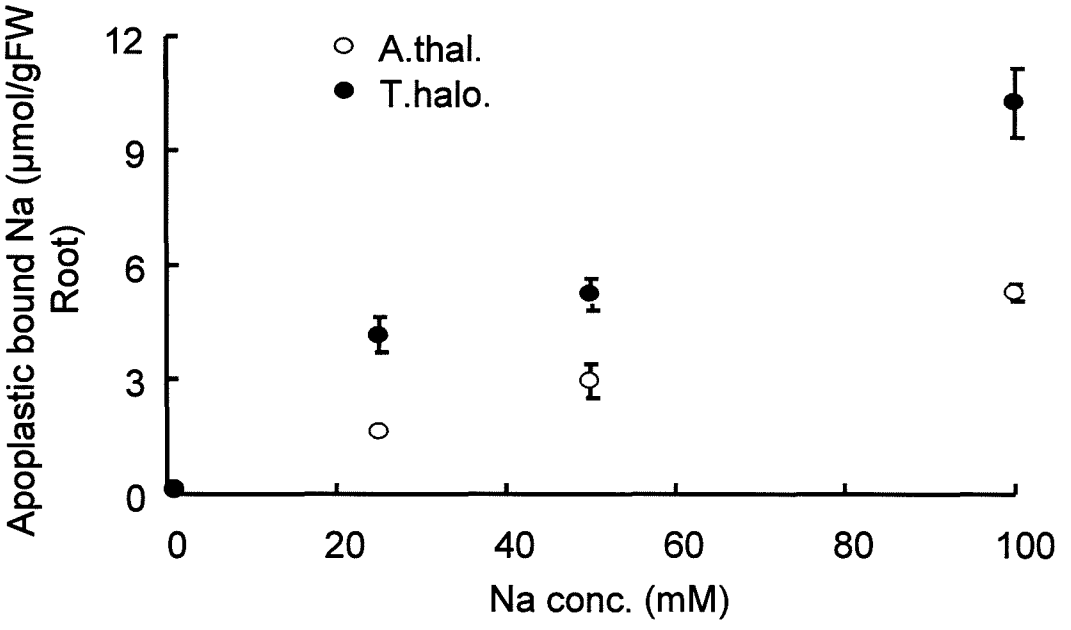

Figure 2-9. Effect of Na concentration in the labelling solution on apoplastic Na binding in boiled roots of Arabidopsis (open circle) and Thellungiella (closed circle). Residual apoplastic Na in the boiled roots after standard washing procedure is plotted against Na concentrations of the ^{22}Na labelled solutions. Values are mean ± SE (n=4).

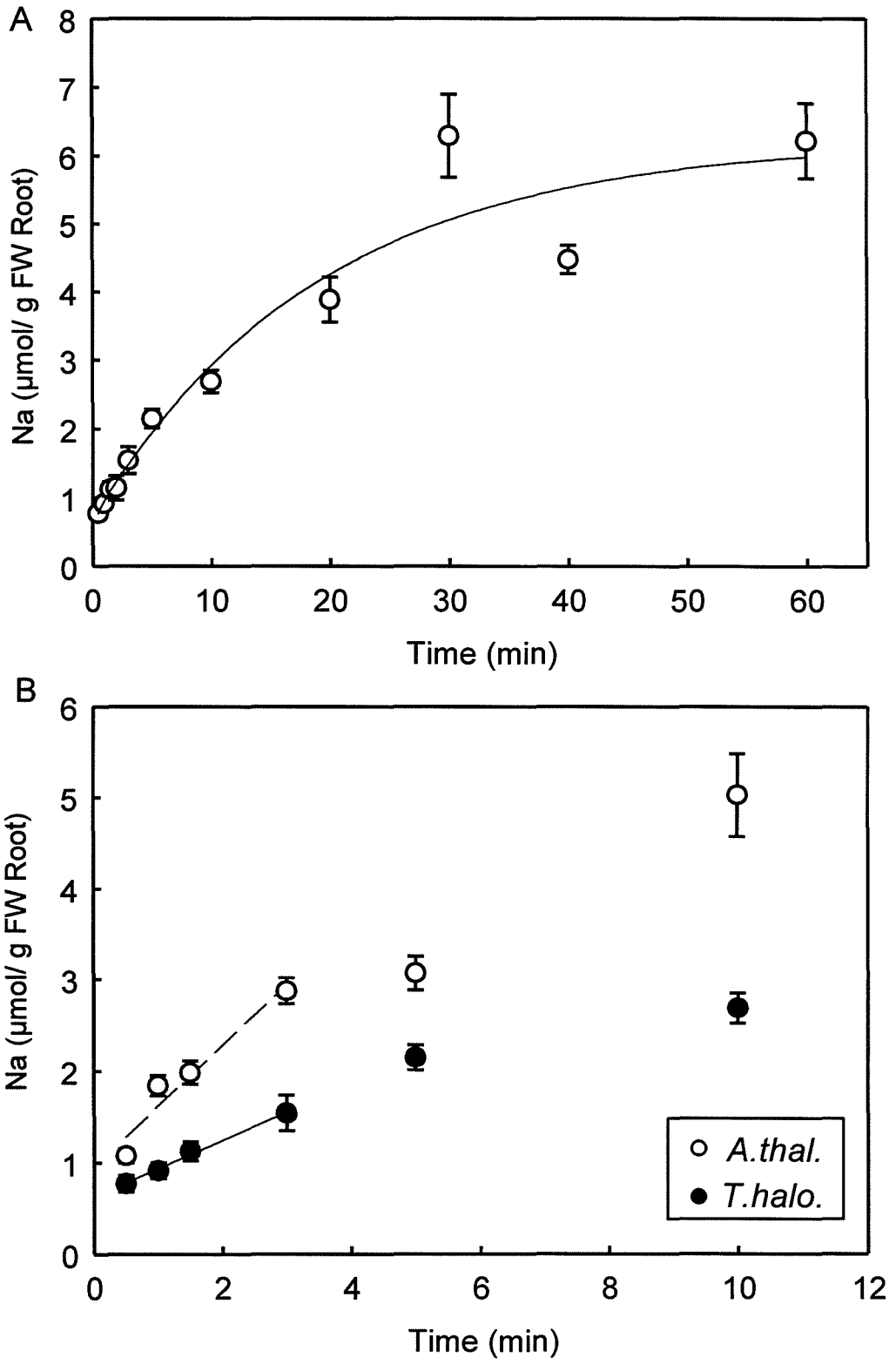

Figure 2-10. Kinetics of steady state unidirectional Na influx into roots of Arabidopsis (open circles) and Thellungiella (closed circles) determined from ^{22}Na accumulation in roots of individual plants from ^{22}Na labelled nutrient solution with 100 mM NaCl and 0.1 mM $CaCl_2$. Values are the mean ± SE (n=4). A. Time course of ^{22}Na accumulation in roots of Thellungiella. B. Initial unidirectional Na uptake to roots of Thellungiella and Arabidopsis.

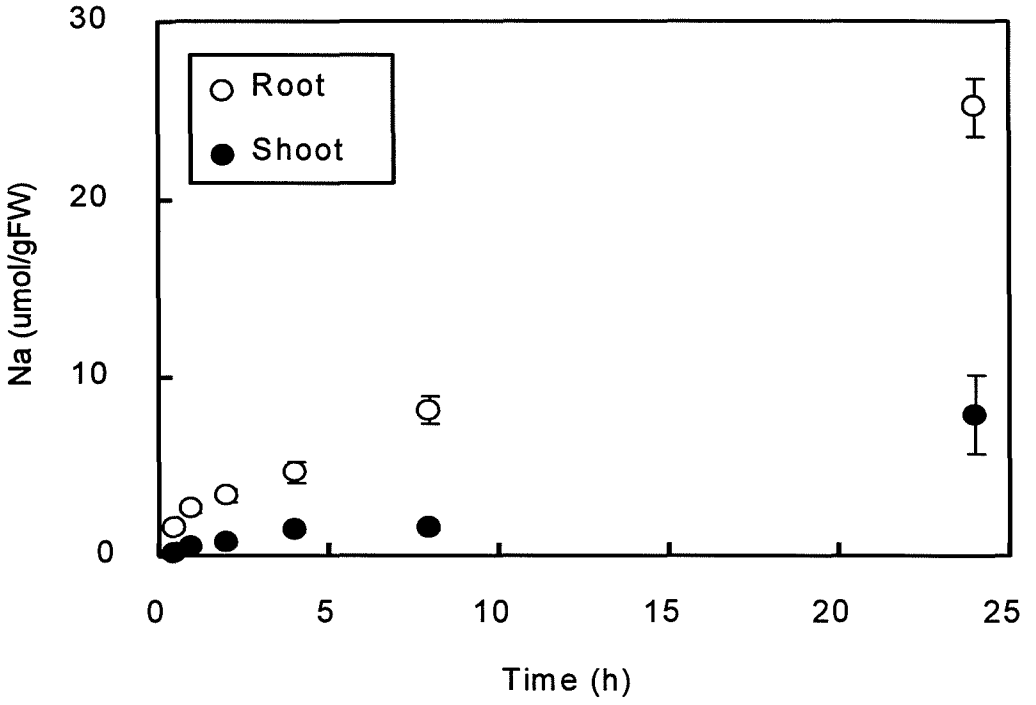

Figure 2-11. Kinetics of Na influx into roots (open circles) and shoots (closed circles) of Thellungiella as determined from ^{22}Na accumulation in the tissues from ^{22}Na labelled nutrient solution with 100 mM NaCl and 0.5 mM $CaCl_2$. Values are the mean ± SE (n=4).

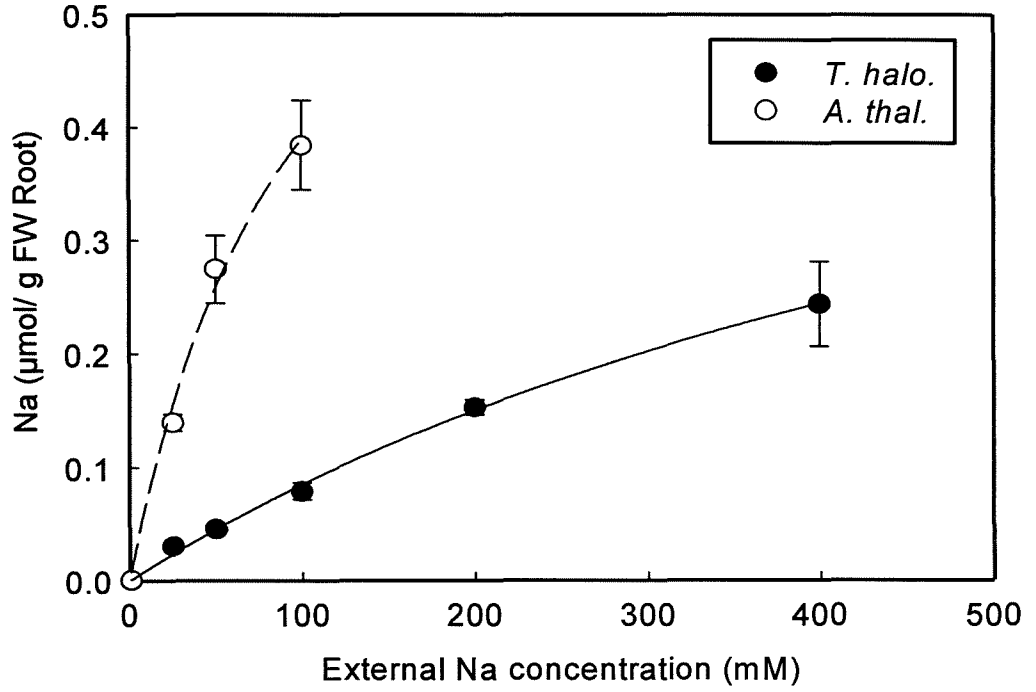

Figure 2-12. Dependence of Na influx into roots of *Arabidopsis* (open circles) and *Thellungiella* (closed circles) on external Na concentration. Na concentration was determined by measuring ^{22}Na accumulation in roots of individual plants after 2 min (Arabidopsis) or 18 min (Thellungiella) labelling in ^{22}Na labelled solution containing 0.1 mM $CaCl_2$ and the indicated amount of NaCl. Values are the mean ± SE (n=4).

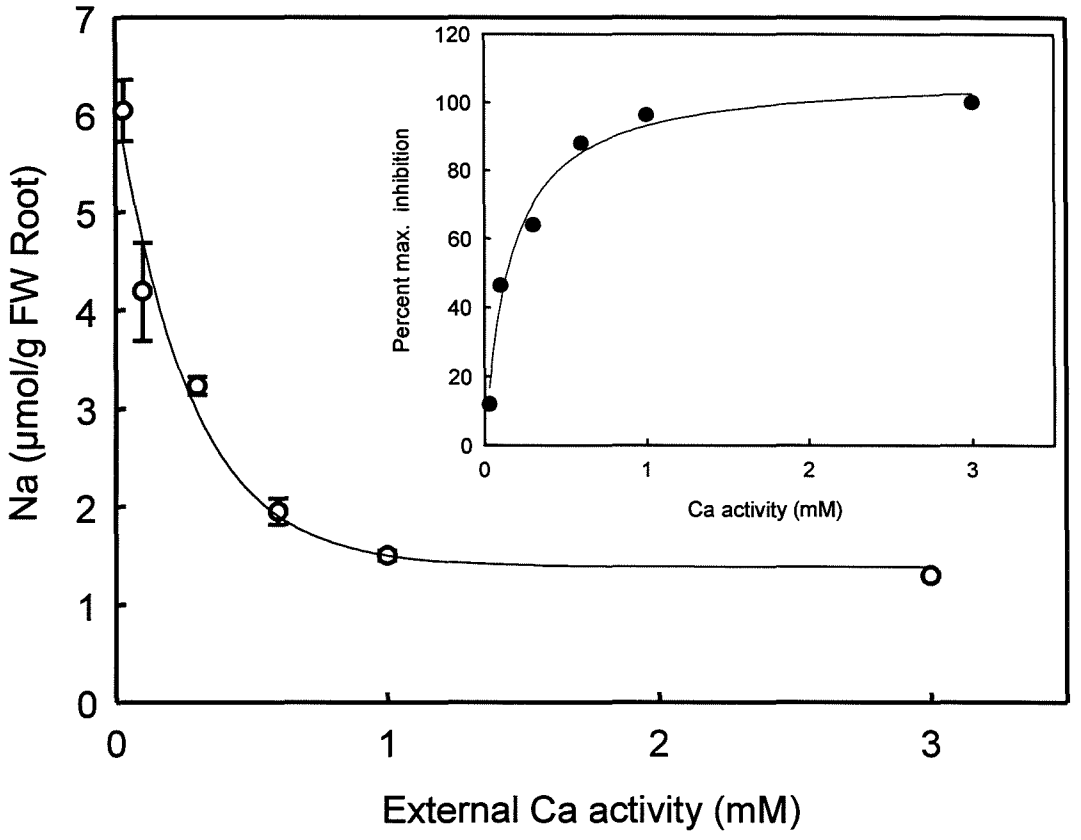

Figure 2-13. Effect of external Ca on Na influx into roots of Thellungiella. Na influx was determined by measuring tracer ^{22}Na level in roots of individual plants after 15 min labelling in ^{22}Na labelled growth solution with 100 mM NaCl and various Ca activities. Values are the mean ± SE (n=4).

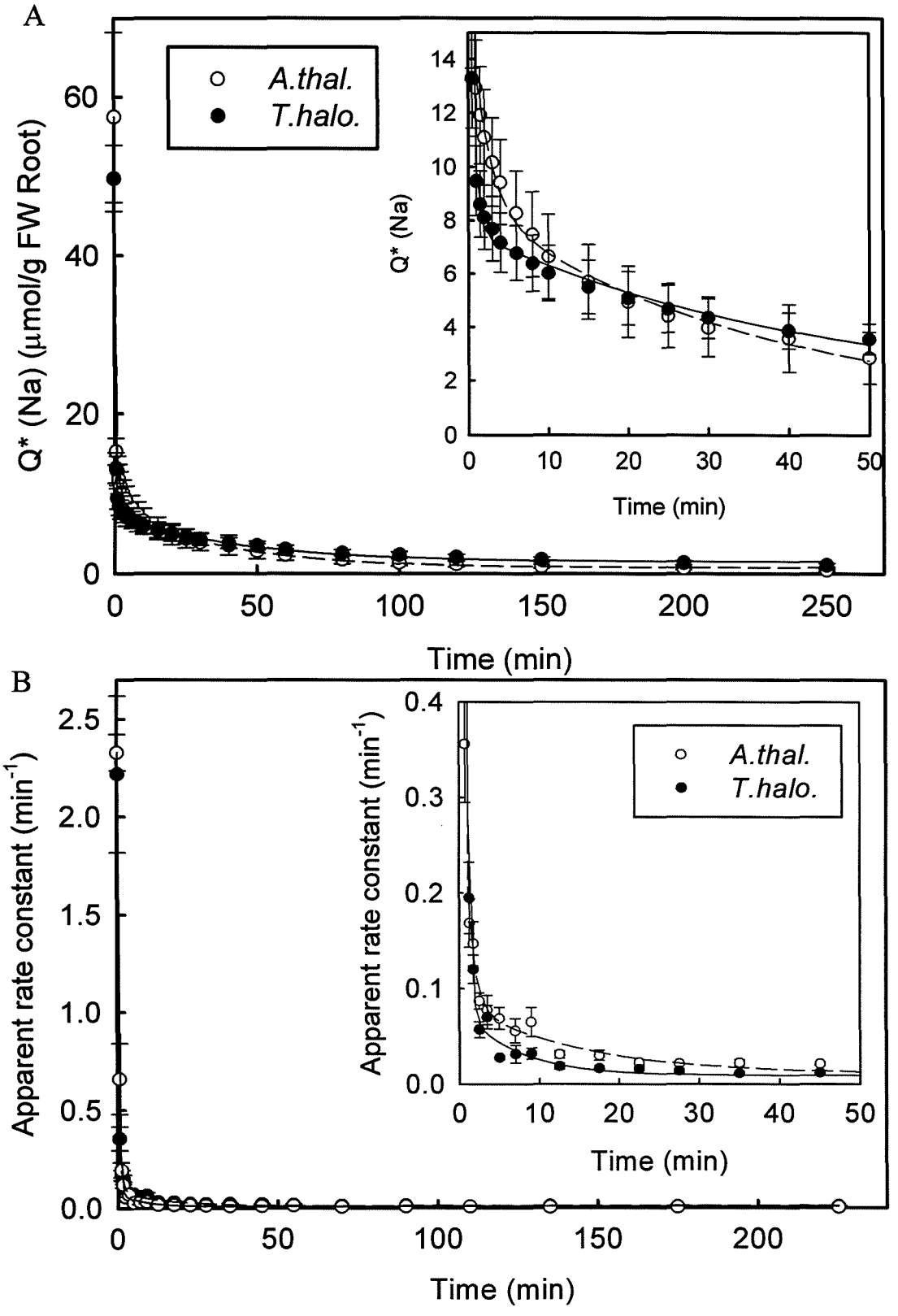

Figure 2-14. Kinetics of Na efflux from roots of *Arabidopsis* (open circle) and *Thellungiella* (close circle). A. Na concentrations of individual plants were determined by measuring tracer ^{22}Na level in aliquots of the efflux solution at a series of time points and in root samples at the end of the experiment. B. Apparent rate constants were calculated according to MacRobbie (1981). Values are the mean ± SE (n=3).

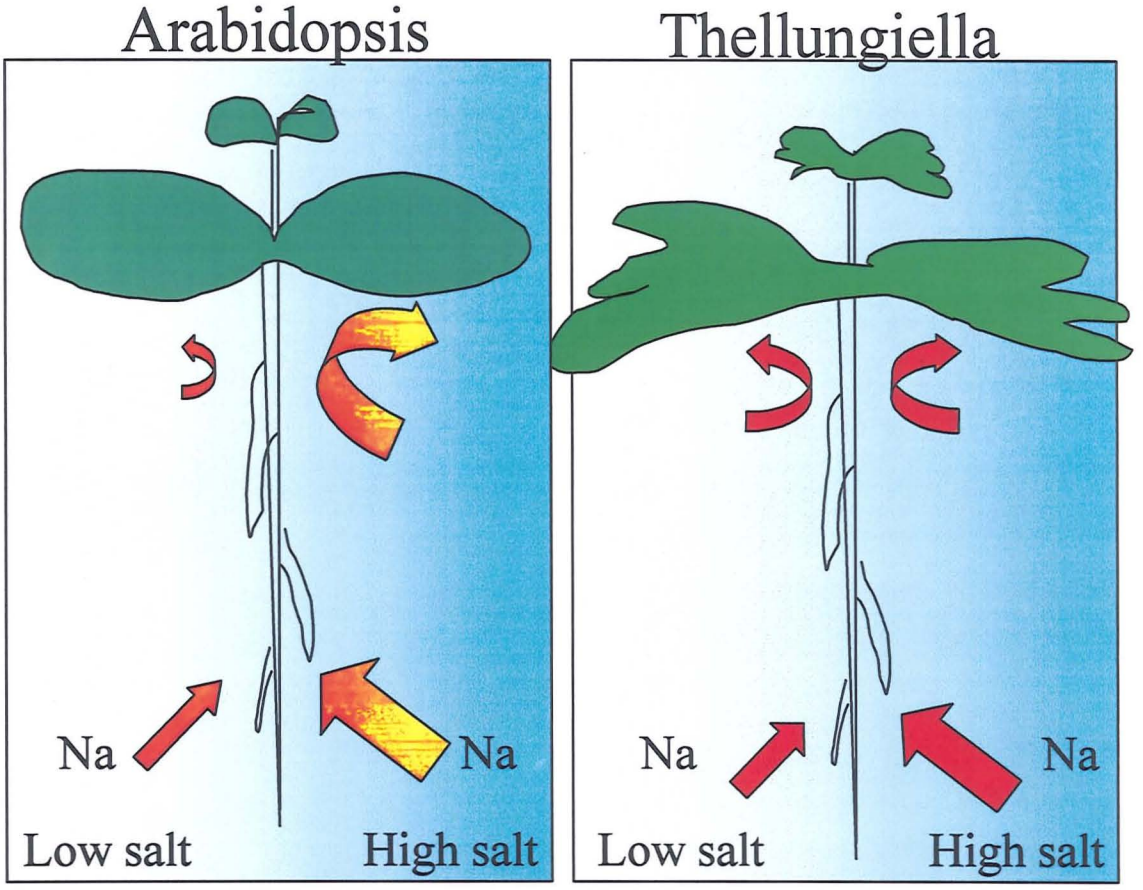

Figure 2-15. Difference of Na allocation between Arabidopsis and Thellungiella before (Low salt) and after 25 h salt treatment in MNS with 100 mM NaCl (High salt).

Table 2-1. The macronutrients in the minimum nutrient solution (MNS) for hydroponic growth of Arabidopsis and Thellungiella.

Macronutrient	Stock Concentration	Volume / l Growth Solution	Final Concentration
KNO_3	1.25 M	1 ml	1.25 mM
$Ca(NO_3)_2$	0.5 M	1 ml	0.5 mM
$MgSO_4$	0.5 M	1 ml	0.5 mM
FeNaEDTA	42.5 mM	1 ml	42.5 μM
KH_2PO_4	0.625 M	1 ml	0.625 mM
NaCl	2 M	1 ml	2 mM

Table 2-2. The micronutrients in the minimum nutrient solution (MNS) for hydroponic growth of Arabidopsis and Thellungiella.

Reagent	Stock Concentration (1000 ×)	Final Concentration (1 ×)
$CuSO_4$	160 μM	0.16 μM
$ZnSO_4$	380 μM	0.38 μM
$MnSO_4$	1.8 mM	1.8 μM
H_3BO_3	45 mM	45 μM
$(NH_4)_6Mo_7O_{24}$	15 μM	0.015 μM
$CoCl_2$	10 μM	0.01 μM

Table 2-3. The final concentrations of the elements in the ×1 standard solution for ion analysis

Element	K	Na	Ca	Mg	S	P
Concentration (mM)	0.51	0.11	0.10	0.041	0.063	0.099

Table 2-4. Compositions of pretreatment and labelling solutions and labelling times used in ^{22}Na fluxes experiments.

Experiment	Pretreat/labelling Solution	Labelling time (min)
Influx kinetics	MNS (-Ca) + NaCl (100 mM) + $CaCl_2$ (0.1 mM)	0.5, 1, 1.5, 3, 5, 10, 30, 40, 60
Ca inhibition	MNS (-Ca) + 100 mM NaCl + various amounts of $CaCl_2$ resulting in Ca activities of 0.03, 0.1,0.6, 1 and 3 mM	15
Na dependence	MNS (-Ca) + various amounts of NaCl (1, 25, 50 100, 200, 400 mM) + $CaCl_2$ (3 mM Ca activity)	2 (*A.t.*) 18 (*T.h.*)
Blockers	Control: same as for influx kinetics Cs: Control + 5 mM CsCl TEA: Control + 20 mM TEA-Cl pH4: Control + 5 mM MES, pH4.1	0.5 and 5
Binding kinetics	MNS + NaCl (100 mM)	1, 2, 5, 10 and 20
Rinse kinetics	Same as for influx kinetics	10
Ca effect on binding	MNS (-Ca) + 100 mM NaCl + various amounts of $CaCl_2$ (0.1, 0.5 and 10.5 mM)	10
Na dependence of apoplastic binding	MNS + various amounts of NaCl (2, 25, 50, 100 mM) + $CaCl_2$ (10mM)	15
Efflux kinetics	Same as for influx kinetics	18 to 24 h

Table 2-5. Compositions of rinse solutions used in ^{22}Na fluxes experiments.

Experiment	Rinse solution	Rinse time (min)
Rinse optimisation	**Standard**: MNS + 100 mM NaCl + 10 mM $CaCl_2$ **+La**: 100 mM NaCl 10 mM $CaCl_2$ 2 mM $LaCl_3$ 5 mM MES, pH4.1 **+Gd**: 100 mM NaCl 10 mM $CaCl_2$ 5 mM $GdCl_3$ 5 mM MES, pH4.1 **Ca**: 75 mM Ca Cl_2 **K**: 100 mM KCl	3 and 10
Rinse kinetics Long-term root/shoot influx kinetics Ca inhibition of influx Na dependence of influx	**Standard**	0.5 to 5 3 3 3
Root influx kinetics Ca effect on binding Na dependence of binding Blocker effects Efflux kinetics	**+La**	3

Table 2-6. Direction and significance of differences in net Na accumulations between treatments or species. Symbols in brackets indicate whether the ratio was larger (>) or smaller (<) than 1 or not significant (=). Numbers are p values obtained in t tests, where pairs represented plants batches grown and treated in parallel, and replicates represented consecutively grown and treated plant batches. Significant p values are in bold. For further explanation see footnotes.

	Shoot				**Root**			
	Comparison between treatments (Salt / control)		Comparison between species (Thellungiella / Arabidopsis)		Comparison between treatments (Salt / control)		Comparison between species (Thellungiella / Arabidopsis)	
	A.thal.	T.halo.	'Control'	'+Na'	A.thal.	T.halo.	'Control'	'+Na'
Short term								
Absolute Na levels	**0.0110 >** [(1)]	**0.0398 >**	**0.0345 >** [(2)]	0.6140 =	**0.0010 >**	**0.0015 >**	0.1191 =	**0.0314 <**
Relative difference	**0.0135 >**	**0.0235 >**	set to =	**0.0135 <**	**0.0082 >**	**0.0097 >**	set to =	0.3974 =
K/Na ratio	**0.0010 >**	**0.0259 <**	**0.0288 <**	0.1260 =	**0.0017 <**	0.2229 =	0.4216 =	**0.0022 >**
Long term								
Absolute values	**0.0019>**	**0.0045 >**	0.4022 =	0.1047 =	**0.0357 >**	**0.0047 >**	0.0860 =	0.1217 =
Relative changes	**0.0029 >**	**0.0524 >**	set to =	**0.0114 <**	**0.0106 >**	**0.0011 >**	set to =	0.2605 =
K/Na ratio	**0.0029<**	**0.0136<**	0.2914 =	**0.0503 >**	**0.0083 <**	**0.0019 <**	0.0913 =	0.2216 =

(1) Read as: Salt treated Arabidopsis plants had a signifcantly higher absolute Na content than control Arabidopsis plants with a p value of 0.011.

(2) Read as: In control conditions Thellungiella had a significantly higher absolute Na content than Arabidopsis.

Table 2-7. Direction and significance of differences in Na shoot/root ratios between treatments or species. Symbols in brackets indicate whether the ratio was larger (>) or smaller (<) than or equal (=) to 1. Numbers are p values obtained in t tests where pairs represented plants batches grown and treated in parallel, and replicates represented consecutively grown and treated plant batches. Significant p values are in bold.

Shoot/Root ratio	Comparison between treatments (Salt / control)		Comparison between species (Thellungiella / Arabidopsis)	
	A.thal.	T.halo.	'Control'	'+Na'
Short term	**0.0102** <	0.1682 =	0.1763 =	0.1351 =
Long term	0.7520 =	0.1209 =	0.9711 =	0.1036 =

Table 2-8. Levels of apoplastic Na in roots of Thellungiella after various washing conditions

	Labelling (µmol/g FW)		
Rinse solution	Rinse time	Medium (0.5 mMCa)	Medium (10 mMCa)
+La	3 min	6.92	8.93
	10 min	5.78	NA
+Gd	3 min	7.74	NA
	10 min	8.44	NA
+Ca	3 min	7.10	11.13
Ca	3 min	6.93	8.29
K	3 min	7.79	NA

Table 2-9. Levels of apoplastic Na in roots of Arabidopsis after various washing conditions

	Labelling (µmol/g FW)		
Rinse solution	Rinse time	Medium (0.5 mMCa)	Medium (10 mMCa)
+La	3 min	3.13	3.70
	10 min	3.73	1.95
MNS	3 min	1.90	NA
+ 100 mM NaCl	10 min	2.46	NA

Table 2-10. Kinetic analysis of ion accumulation and fluxes: fitted equations and parameters.

Parameters extracted	Fitted equation	R^2	Fig.
Net Na uptake to the shoot (h)	*At*: $f(t) = 2.8 + 28.7\ (1 - e^{-0.015\ t})$	0.99	2-5A
	Th: $f(t) = 5.2 + 5.1(1 - e^{-0.058\ t})$	0.99	
Net Na uptake to the root (h)	*At*: $f(t) = 1.10 + 8.95\ (1 - e^{-0.20t}) + 10.67\ (1 - e^{-0.03t})$	0.99	2-5B
	Th: $f(t) = 0.79 + 7.13\ (1 - e^{-0.42t}) + 1.99\ (1 - e^{-0.06t})$	0.99	
Na influx time curve (min)	$f(t) = 0.68\ (1 - e^{-4.86t}) + 5.55\ (1 - e^{-0.05t})$	0.93	2-10A
Unidirectional Na influx (μmol gFW^{-1} min^{-1})	*At*: $f(t) = 0.95 + 0.66\ t$	0.94	2-10B
	Th: $f(t) = 0.62 + 0.31\ t$	0.99	
Na affinity of influx	*At*: $f(c) = 0.79\ c / (102 + c)$	0.99	2-12
	Th: $f(c) = 0.66\ c / (671 + c)$	0.99	
Ca inhibition of influx K_i (mM)	$f(c) = 1.4 + 4.9\ e^{-3.7\ c}$	0.97	2-13
	inset: $f(c) = 100\ c / (0.16 + c)$	0.98	
Na efflux (min)	*At*: $f(t) = 41\ e^{-36586\ t} + 7.9\ e^{-0.45\ t} + 7.8\ e^{-0.03\ t} + 0.77$	0.99	2-14A
	Th: $f(t) = 29\ e^{-3898\ t} + 13\ e^{-1.67\ t} + 6.1\ e^{-0.02\ t} + 1.5$	0.99	

Table 2-11. Effects of putative blockers on Na influx into roots of Arabidopsis and Thellungiella. Effects of 5 mM CsCl, 20 mM TEA-Cl and 5 mM MES (pH 4.1) on the Na influx over the first 5 min after addition of blockers to growth solution with 100 mM NaCl and 0.1 mM $CaCl_2$ are presented as influx relative to control (% influx in control medium ± SEM, n = 4).

	A. thal.	*T. halo.*
5 mM Cs	135.44 ± 2.73	161.01 ± 3.59
20 mM TEA	192.11 ± 2.42	156.62 ± 4.74
5 mM MES (pH 4.1)	94.55 ± 1.95	142.47 ± 2.44

Table 2-12: Quantitative comparison of the measured unidirectional influx with those of net Na uptake into the plants. The top panel calculated the unidirectional influx of Na in 25 h using the rate from ^{22}Na flux experiment. The bottom panel calculated total net Na uptake in 25 h using the data from net Na concentration measurement. The proportion of net Na uptake in the total unidirectional Na influx were estimated for both Arabidopsis and Thellungiella. The FW/DW ratios and shoot/root ratios are estimated from previous experimental data.

	Influx umol/min gFW	Influx ug/min gFW	FW/DW root	Influx ug/min gDW	**Influx in 25 h mg/g DW**
A.thal	0.66	15.18	20	303.6	**455.4**
T. halo	0.31	7.13	12	85.56	**128.3**
	Net SNa 25 h mg/gDW	Net RNa 25 h mg/gDW	root+shoot	**Net plant Na 25 h mg/gDW**	**Net/uni %**
A.thal	12	18	1+7	**102.0**	**22.4**
T. halo	5.5	10	1+3.5	**29.3**	**22.8**

Chapter 3 Homeostasis of other ions under salt stress

3.1 Introduction

3.1.1 ICP-OES

The most common method to measure tissue ion contents during the last decades was atomic absorption. For each element it measures the absorption at a characteristic wavelength on a full spectrum light background. Each run can only measure one element making ion content measurements time-consuming and laborious work.

In this study, ion contents were measured with Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES). ICP-OES is a relatively recent technique for measuring several elements simultaneously with fairly low detection limit. Liquid sample in water or diluted acid is fed into the ICP as a very fine spray through a nebulizor. Each atom or ion is excited by the tip of a flame under the protection of argon gas to emit electrons or photons at characteristic wavelengths. One or several optical spectrometers can scan a wide range of wavelengths within a short time. It enables many elements to be monitored in one scan by measuring intensities at characteristic wavelengths across a full spectrum. Using a set of multi-element standards, standard curves plotting light intensities against ion concentrations can be drawn at characteristic wavelengths of each target elements.

ICP has generally low detection limits and a good dynamic range. For example, K can be detected from at least 0.01 mg/l up to 30 mg/l. Samples prepared from Arabidopsis and Thellungiella dried tissue with a dilution factor of 5000× (w: v) detect abundant ions such as K, Na, Ca, Mg, P and S, as well as micronutrients such as Zn, B and Fe. Unfortunately detection of C, N and O is insufficient since C, N and O in the air tend to interfere with sample readings. Detection of Cl is very weak because chlorine is not easy to excite in a plasma source and its emission wavelengths are challenging for

conventional optics, e.g. chlorine wavelengths that yield good sensitivity are low (133 nm and 134 nm) and it is not possible to measure them with conventional solidstate detectors or photomultiplier tubes. Therefore although Cl might play an important role in salt toxicity, Cl contents were not analysed in this project.

3.1.2 Questions addressed

3.1.2.1 Ion profiles

ICP-OES allows us to collect ion profiles from the shoots and roots of Arabidopsis and Thellungiella after different periods of salt treatment, and therefore can detect any changes in uptake and allocation of important elements in response to salt stress. The obtained profiles will also be useful for comparison with other ionic stresses (e.g. K, Ca deficiency) and abiotic stresses (e.g. drought and cold stress). Furthermore, ion profile data provided background information for interpreting transcriptional expression profiles of membrane transporters before and after salt treatment in Arabidopsis and Thellungiella. For example, expression profiles of Ca and Mg transporters can be correlated with tissue accumulation of these ions. Na dependent phosphate transporters are present in yeast, bacteria and some sea grass species (Rubio et al., 2004), and genes for putative Na dependent phosphate transporters are also present in the Arabidopsis genome. Data on P (and S) concentrations in Arabidopsis and Thellungiella plants during salt stress could provide clues to the effect of salt on the regulation of transporters for these ions.

3.1.2.2 Effect of K and Ca on ion profiles during salt stress

Because of the similar physico-chemical properties of K and Na, it has been suggested that Na and K compete for the same transport pathways. Maintenance of high tissue K/Na ratio was suggested to improve salt tolerance in plants (Maathius and Amtmann,

1999). Ca is an important macronutrient for plant growth and an important cell signalling component for various stress responses. Additional Ca is well known to improve plant salt tolerance (Lahaye and Epstein, 1969; Lauchli, 1990b). I therefore measured tissue ion profiles when additional K or Ca was supplied during salt treatment.

3.1.3 Experimental design

Plant growth and salt treatments were generally the same as for Na concentration measurements described in Chapter 2. In addition, a greater range of salt treatments with different concentrations of K and Ca were applied to Arabidopsis and Thellungiella to reveal the effects of K and Ca on ion accumulation during salt stress.

3.2 Materials and methods

3.2.1 Plant growth

Arabidopsis and Thellungiella plants were grown hydroponically as described in Chapter 2 (Section 2.2.1).

3.2.2 Treatments and harvesting

Arabidopsis and Thellungiella plants were grown hydroponically in MNS for 4 weeks before being subjected to various experimental treatments. Short-term and long-term salt treatments were applied to both Arabidopsis and Thellungiella plants as described in Chapter 2 (section 2.2.2). Shoots and roots were harvested separately and analysed as described in Chapter 2 (section 2.2.2).

Net ion uptake into roots and shoots of the two species were analyzed after 25 h salt treatment (100 mM NaCl), over a 72 h time course (100 mM NaCl), and after 6 weeks of salt stress (50 or 100 mM NaCl for Arabidopsis and Thellungiella respectively). Additional salt treatments with elevated concentrations of KCl or $CaCl_2$ were applied to both Arabidopsis and Thellungiella for 25 h. Table 3-1 lists the treatments and the

composition of the growth solutions in the short-term salt stress experiment. Each replicate consisted of 6 to 7 plants. 'Control' and '+NaCl' treatments had four replicates, while all the other treatments were repeated three times.

3.2.3 Ion analysis

Protocols of ion extraction and sample dilution, composition of the standard solutions, ICP-OES calibration and sample ion determination were as described in Chapter 2 (Section 2.2.3). ICP-OES measures many ions simultaneously. In this study, contents of two monovalent cations, Na and K, two divalent cations, Ca and Mg, and 2 elements, P and S, taken up by plants as the anions phosphate and sulfate, were analysed. Ion profiles were compared between Arabidopsis and Thellungiella plants.

3.2.4 Data processing and statistical analysis

In short- and long-term salt treatment experiments, ion concentrations were expressed as percent of dry weight (% DW). Relative ion concentrations of the '+NaCl' samples were expressed as percent of 'Control' concentrations (% control). Relative ion concentrations of other salt treated samples were expressed as percent of the concentrations in their specific control samples ('+KCl' for '+NaCl+KCl', '+$CaCl_2$' for '+NaCl+$CaCl_2$').

T tests were applied to compare the concentrations of each ion between Arabidopsis and Thellungiella after a particular treatment and the concentrations of each ion between salt-treated samples and their specific controls for a particular species.

To determine the time course of net ion uptake the ion concentration of each element was plotted against time. Time courses of net ion accumulation were fitted to various kinetic models with SigmaPlot® (Systat Software Inc., Richmond, USA). Interactions between the effects of ion treatments and species on ion concentrations were tested using 3-way ANOVA with MiniTab® statistical analysis software package (MiniTab

Inc., PA, USA). In the test, ion concentrations in a specific tissue is the variant, while species, salt treatment and ion supplement were the 3 factors. The significance of the difference of ion concentration between Arabidopsis and Thellungiella, before and after salt treatment and with and without ion supplement (K or Ca) was analysed. Interference between the above factors on ion concentrations is also tested. P values equal or less than 0.05 were considered significant. For the elements that showed significant differences, a plot of the mean ion concentration of both species in control and salt-treated conditions is presented.

3.3 Results

3.3.1 Ion profiles under control conditions

Figure 3-1 shows ion profiles obtained under control conditions. Most differences between Arabidopsis and Thellungiella appeared in the shoots (Figure 3-1A). As described before the Na concentration of Thellungiella shoots was about twice that of Arabidopsis. Despite the similar physico-chemical properties of K and Na, both plants distinguished between the two ions (similar concentrations in MNS). K concentrations in the shoots were similar between Arabidopsis and Thellungiella, and much higher than concentrations of Na and most other ions. The Ca concentration in the shoots of Arabidopsis was similar to K. Thellungiella plants maintained a much lower Ca level in the shoots than Arabidopsis. Thellungiella also had a lower Mg concentration in the shoots than Arabidopsis. The P concentrations were similar between Arabidopsis and Thellungiella. The S concentration of Thellungiella shoots was slightly higher than that of Arabidopsis shoots. In the roots there was little difference in ion concentrations between the two species except for a lower K concentration of Thellungiella than Arabidopsis (Figure 3-1B). This general profile did not change considerably over the course of several weeks. The profiles of ion concentrations in 10 week old plants were

similar to those in 4 week old plants, but with a lower overall ion concentration in the shoots (data not shown). Root K concentrations were no longer different between Arabidopsis and Thellungiella in the older plants (data not shown). Lower concentrations of divalent cations were partially compensated by higher amounts of Na. K, S and P were equally distributed between root and shoot in both plant species. Other ions were preferentially accumulated in shoots, e.g. Ca and Mg in Arabidopsis, and Na in Thellungiella.

3.3.2 Tissue concentrations of K during salt stress

3.3.2.1 K concentrations after short-term salt treatment

In control conditions, the K concentrations of the shoots did not significantly differ between Arabidopsis and Thellungiella (Figure 3-2A, C). The root K concentration in Arabidopsis was higher than that in Thellungiella (Figure 3-2B, D). The K concentrations of Arabidopsis changed quickly after salt was added in the growth solutions. After 25 h treatment with 100 mM NaCl, the K concentration had decreased significantly in both roots and shoots of Arabidopsis ($p=0.05$ for both roots and shoots, Figure 3-2 and Table 3-2). In Thellungiella, K concentrations remained the same during the 25 h salt treatment. In control conditions the absolute concentration of root K in Arabidopsis was significantly higher than in Thellungiella ($p=0.02$, Figure 3-2 and Table 3-2), but after 25 h salt treatment there was no difference in root K concentrations between the two species due to K loss from Arabidopsis (Figure 3-2 and Table 3-2). The shoot/root ratio of the K concentration was constant in Thellungiella at around 1 (Figure 3-3A and Table 3-7). This ratio was 1/2 in control Arabidopsis plants, and increased slightly to about 2/3 after 25 h treatment in 100 mM NaCl (Figure 3-3A and Table 3-7).

As discussed in Chapter 2 (section 2.3.1), both Arabidopsis and Thellungiella plants maintain very high K/Na ratios in non-saline conditions especially in the roots. After salt treatment for a short period, K/Na ratios in both roots and shoots of Arabidopsis and Thellungiella plants decreased significantly. K/Na ratios after 25 h treatment with 100 mM NaCl were slightly higher in Thellungiella than in Arabidopsis (see Section 2.3.1 for details).

3.3.2.2 K concentrations after long-term salt treatment

After 6 weeks salt treatment K concentrations were similar between Arabidopsis and Thellungiella. Note that this treatment used 100 mM NaCl for Thellungiella and only 50 mM NaCl for Arabidopsis. K concentrations of both root and shoot decreased by about 10 to 20% relative to K concentrations of the control plants in both Arabidopsis and Thellungiella (Figure 3-4C, D), but t tests showed low significance for these changes (Table 3-2). Shoot/root ratios of the K concentration were also similar between Arabidopsis and Thellungiella, at about 3/5 with no changes after long-term salt treatments (Figure 3-3B).

3.3.2.3 Kinetics of change in K concentration during 72 h salt treatment

Kinetics of net ion uptake or loss were investigated by measuring ion concentrations in individual plants treated with 100 mM NaCl for 0, 6, 24 and 72 h. Net changes in K concentrations in response to salt treatment followed similar kinetics in the shoots of Arabidopsis and Thellungiella for at least 72 h (Figure 3-5A). During the first 24 h of salt treatment, the shoot K concentration increased gradually from 35 mg/g shoot DW to 40 mg/g shoot DW in Thellungiella plants. Shoot K concentration of Thellungiella plants decreased after 24 h salt treatment reaching a value of 33 mg/g shoot DW at 72 h. The shoot K concentration in Arabidopsis plants before salt treatment was about 5 mg/g shoot DW lower than K concentration in the shoots of Thellungiella. K concentration

showed a transient decrease during the first 6 h of salt treatment in the shoots of Arabidopsis plants, but was restored after 24 h. Similar to Thellungiella, the K concentration in the shoots of Arabidopsis plants decreased after 24 h treatment reaching a value of 24 mg/g shoot DW at 72 h after onset of salt stress. Between 6 h and 72 h of salt treatment, the difference in K concentrations between the shoots of Arabidopsis and Thellungiella remained constant at about 9 mg/g shoot DW.

In the roots, the kinetics of salt induced net changes in K concentration clearly differed between Arabidopsis and Thellungiella (Figure 3-5B). Arabidopsis root K concentration increased slightly over the first 6 h of salt treatment but then decreased steadily during extended salt treatment. In Thellungiella plants, the K concentration in the roots was lower than in Arabidopsis at the beginning of the salt treatment but it decreased at a much lower rate than in Arabidopsis. The two curves intersect at approximately 26 h after the application of the salt treatment. This result was consistent with the K concentrations measured in the roots of the two species after treatment with 100 mM NaCl for 25 h. Linear regression fits revealed rates of K loss of 0.3 ± 0.06 and 0.1 ± 0.02 mg/g root DW/h in Arabidopsis and Thellungiella respectively (Table 3-8).

3.3.3 Concentrations of Ca and Mg under salt stress

3.3.3.1 Concentrations of Ca and Mg after short-term salt treatment

Shoot Ca concentrations of 4 week old Thellungiella plants growing in control medium were only a third of the Ca concentration in the shoots of Arabidopsis plants (Figure 3-6A). Shoot Ca concentrations in neither Arabidopsis nor Thellungiella were affected by 25 h treatment with 100 mM NaCl (Figure 3-6A, C). In the roots, the absolute Ca concentrations were similar between control plants of Arabidopsis and Thellungiella (Figure 3-6B). Ca concentrations in the roots decreased significantly in both species after 25 h salt treatment (Figure 3-6B, D, P=0.01 for Arabidopsis, P=0.003 for

Thellungiella, Table 3-3). The root Ca concentration of salt treated Arabidopsis plants was 75% of the control level (Figure 3-6D). After the 25 h salt treatment, the root Ca concentration in Thellungiella plants was 63% of the control level (Figure 3-6D). The difference between relative changes of the root Ca concentrations between Arabidopsis and Thellungiella after salt treatment was not considered significant by t-test (Table 3-3). Shoot/root ratios of Ca concentration were significantly different between Arabidopsis and Thellungiella (Figure 3-7A). In control plants, the shoot/root ratio of Ca concentrations was 8/1 in Arabidopsis, and about 3/1 in Thellungiella. After 25 h salt treatment, the shoot/root ratio of Ca concentration in Arabidopsis increased slightly to 8.5/1, and this increase was considered significant by t-test (P=0.05, Table 3-7). In Thellungiella plants, Ca shoot/root ratio also increased, but not significantly to about 4/1 after short-term salt treatment (Table 3-7).

The overall picture of Mg concentrations was quite similar to that of Ca concentrations in both species. The shoot Mg concentration of Thellungiella plants was about half of the shoot Mg concentration of Arabidopsis plants (Figure 3-9A). Shoot Mg concentrations were not affected by short-term salt stress in either species (Figure 3-9A, C). Root Mg concentrations in the control plants were similar between Arabidopsis and Thellungiella (Figure 3-9B). After 25 h salt treatment, Mg concentrations in the roots decreased in both species (Figure 3-9B, D). Mg concentration in the roots of Arabidopsis plants after treatment was about 70% of the control level (Figure 3-9D). This decrease in root Mg concentration for Arabidopsis was considered significant by t-test (P=0.01, Table 3-4). Mg concentration in the roots of Thellungiella after salt treatment was 78% of the control level (Figure 3-9D, P=0.04, Table 3-4). Similar to Ca, the shoot/root ratios of Mg concentration were higher in Arabidopsis than in Thellungiella (Figure 3-8A). The Mg shoot/root ratio increased slightly in Arabidopsis

after short-term salt stress (Figure 3-8A), from 5.5/1 to 7/1, but this increase was not significant (Table 3-7). The Mg shoot/root ratio in Thellungiella was 2/1 in control plants and increased slightly to 2.5/1 after 25 h salt treatment but this increase was not significant.

3.3.3.2 Concentrations of Ca and Mg after long term salt treatment

Ca concentrations in 10 week old control plants were much lower than in younger plants. The shoot Ca concentration of Thellungiella was half of that in Arabidopsis (Figure 3-10A). Root Ca concentrations of control plants were similar in Arabidopsis and Thellungiella (Figure 3-10B). After 6 weeks salt treatment, the shoot Ca concentration of Thellungiella plants was significantly lower than that of the control plants (Figure 3-10A, P=0.009, Table 3). The decrease in shoot Ca concentration of Arabidopsis after salt treatment was not significant (Table 3-3). Ca concentrations in the roots after salt treatment for 6 weeks were significantly lower than the control levels in both species (Figure 10B, P=0.05 for Arabidopsis, P=0.01 for Thellungiella, Table 3-3). Ca concentrations after salt treatment in both roots and shoots of Thellungiella were significantly lower than those of Arabidopsis (Figure 10, P=0.05 for roots, P=0.03 for shoots, Table 3-3). The shoot Ca concentration of Arabidopsis decreased by about 20% (Figure 3-10A). The shoot Ca concentration of Thellungiella after salt treatment was only about 30% of the control level (Figure 3-10A). However the difference in the relative changes of shoots Ca concentrations was not significant in t test due to large standard errors (Table 3-3). The decrease in root Ca concentration of Arabidopsis after 6 weeks salt treatment was similar to the decrease in shoot Ca concentration, which was about 20% of the control level (Figure 3-10 C, D). The root Ca concentration of Thellungiella after long-term salt treatment was about 2/3 of the control level (Figure 3-10D). T-test suggested that the relative change of root Ca concentrations was

significantly different between Arabidopsis and Thellungiella (P=0.01, Table 3). Shoot/root ratios of Ca concentration in both Arabidopsis and Thellungiella were lower in 10 week old plants than in the younger plants. This ratio in Arabidopsis was not affected by long-term salt treatment (Figure 3-8B). Arabidopsis maintained a Ca shoot/root ratio of about 3.5/1 after long-term salt stress. The shoot/root ratio of Ca concentration of Thellungiella was lower than for Arabidopsis. Thellungiella did not maintain Ca shoot/root ratios at a constant level under salt stress. This ratio in Thellungiella decreased from 2/1 to 1/1 after 6 weeks treatment with 100 mM NaCl.

The changes in Mg concentrations in 10 week old plants were again similar to the changes in Ca concentrations. Mg concentrations in older plants were lower than in younger plants of both species. The shoot Mg concentration of control Thellungiella was about half of that in Arabidopsis (Figure 3-11A). Shoot Mg concentrations decreased after long-term salt treatment in both plants (Figure 3-11A, C) to about 69% of the control level in Arabidopsis and 28% of the control level in Thellungiella (Figure 3-11C). However, in neither species was the decrease in shoot Mg concentration considered significant by t-tests (Table 3-4). By contrast, t-tests did show significant decreases of root Mg concentrations in both Arabidopsis and Thellungiella after long-term salt treatment (P=0.004 for Arabidopsis and P=0.008 for Thellungiella, Table 3-4). The root Mg concentration after salt treatment was 55% of the control level in Arabidopsis plants, and 60% in Thellungiella (Figure 3-11D). The shoot/root ratio of Mg concentration in 10 week old plants was lower than in the younger plants of both species. Arabidopsis plants had a higher shoot/root ratio of Mg concentration than Thellungiella (Figure 3-9B, P=0.03, Table 3-7). The shoot/root ratio of Mg concentration did not significantly change after long-term salt stress in Arabidopsis (Figure 3-9B). This ratio was 2/1 in control Arabidopsis, and 2.5/1 after treatment. The

Mg shoot/root ratio of Thellungiella decreased from 1.2/1 to 0.6/1 after long-term salt treatment. However this decrease was not considered significant by t-tests (Table 3-7).

3.3.3.3 Kinetics of changes in Ca and Mg concentrations during 72 h salt treatment

The Ca concentration in the shoots of Arabidopsis plants decreased slightly during 72 h treatment in 100 mM NaCl (Figure 3-12A). The decrease in shoot Ca concentration of Thellungiella plants was less pronounced than in Arabidopsis (Figure 3-12A). The shoot Ca concentration in Arabidopsis decreased from 27 mg/g shoot DW to 22 mg/g shoot DW during the first 6 h treatment, but was restored after 24 h. The Ca concentration in the shoots of Arabidopsis decreased again after 24 h salt treatment. After 72 h treatment the Ca concentration in the shoots of Arabidopsis was about 18 mg/g shoot DW. During the initial 24 h of salt treatment, the shoot Ca concentration in Thellungiella plants remained between 12 and 13 mg/g shoot DW. The decrease of shoot Ca in Thellungiella started after 24 h. The shoot Ca concentration for Thellungiella was about 9.6 mg/g shoot DW after 72 h salt treatment. Root Ca concentrations in both Arabidopsis and Thellungiella decreased sharply during the first 6 h of salt treatment (Figure 3-12B). Between 6 and 72 h, the root Ca concentration in Arabidopsis plants was fairly constant, whereas Ca concentration in the roots of Thellungiella continued to decrease albeit very slowly (Figure 3-12B).

Kinetics of the changes in Mg concentration in the shoots of Arabidopsis and Thellungiella resembled those of Ca. In the shoots of Arabidopsis the Mg concentration decreased in the first 6 h from 7.6 mg/g shoot DW to 6.7 mg/g shoot DW, and was restored at 24 h after salt application (Figure 3-13A). The shoot Mg concentration in Arabidopsis 72 h after the salt application was 5.8 mg/g shoot DW (Figure 3-13A). The shoot Ca concentrations of Thellungiella plants were always about 3 mg/g shoot DW

lower than those of Arabidopsis (Figure 3-13A). The shoot Mg concentration in Thellungiella decreased steadily from 4.5 mg/g shoot DW to 3 mg/g shoot DW during the 72 h of salt treatment (Figure 3-13A). The decrease in shoot Mg concentration of Thellungiella could be fitted with a single exponential decay model (Table 3-8). Root Mg concentrations of Arabidopsis and Thellungiella followed the changes of those in the shoots of Thellungiella (Figure 3-13B). Root Mg concentrations of Thellungiella were always about 0.5 mg/g root DW higher than those of Arabidopsis (Figure 3-13B).

3.3.4 Tissue concentrations of P and S during salt stress

3.3.4.1 Concentrations of P and S after short term salt treatment

P concentrations of both shoots and roots of control plants were lower in Thellungiella than in Arabidopsis (Figure 3-14, P=0.01 for shoots, P=0.003 for roots, Table 3-5). P concentrations of both shoots and roots of Arabidopsis decreased significantly by about 10% after the 25 h salt treatment (Figure 3-14C, D, P=0.02 for shoots, P=0.0002 for roots, Table 3-5). By contrast, P concentrations in Thellungiella plants remained unchanged after salt treatment (Figure 3-14). The shoot/root ratio of P concentration in control Thellungiella was slightly lower than in Arabidopsis (Figure 3-15A), but this was not significant (P= 0.07, Table 3-7). Arabidopsis maintained a P shoot/root ratio of about 7/10 under short-term salt stress. The shoot/root ratio of P concentration of Thellungiella increased from slightly less than 0.6/1 to 0.66/1, but this was not significant (Table 3-7).

The shoot S concentration of Thellungiella was slightly higher than that of Arabidopsis shoots (Figure 3-15A), but the difference was not significant (Table 3-6). After the 25 h salt treatment, S concentrations in the shoots of both Arabidopsis and Thellungiella remained unchanged (Figure 3-15A, C). Thellungiella plants had lower root S concentration than Arabidopsis in control conditions (Figure 3-15B, D, P=0.04, Table 3-

6). After short-term salt treatment, root S concentration in Arabidopsis decreased by about 25% (Figure 3-15D), but this decrease was not significant (Table 3-6). The shoot/root ratio of S concentration was higher in Thellungiella than in Arabidopsis in control conditions (Figure 3-17B, P=0.01, Table 3-7). S shoot/root ratio in neither species was altered by short-term salt treatment (Figure 3-17B). This ratio was about 1/2 in Arabidopsis, and 1/1 in Thellungiella.

3.3.4.2 Tissue concentrations of P and S after long-term salt treatment

P concentrations in 10 week old plants were similar to those measured in the younger plants of both species. The shoot P concentration of Arabidopsis after 6 weeks treatment in 50 mM NaCl was slightly higher than that of the control plants (Figure 3-18A, C), but the difference was not significant (Table 3-5). The root P concentration of Arabidopsis did not change after the long-term salt treatment (Figure 3-18B, D). After 6 weeks treatment with 100 mM NaCl the P concentration did not change in either roots or shoots of Thellungiella (Figure 3-18). The shoot P concentration of control plants was slightly lower in Thellungiella than in Arabidopsis (Figure 3-18A), whereas the root P concentration of control plants was slightly higher in Thellungiella than in Arabidopsis (Figure 3-18B). Neither of these differences was significant (Table 3-5). The shoot/root ratio of the P concentration in Arabidopsis increased after long-term salt treatment from about 0.67/1 to 1/1 (Figure 3-16B). The p value of this increase was just below the significance threshold (P=0.059, Table 3-7). In Thellungiella the P shoot/root ratio of 10 week old control plants was lower than that of younger plants (Figure 3-16). The P shoot/root ratios was not altered by the long-term salt treatment (Table 3-7). The older Thellungiella plants maintained a shoot/root ratio of P of around 0.5/1 independent of the salt concentration in the medium.

In control conditions S concentrations of 10 week old Arabidopsis plants were lower than those in the younger plants. Shoot S concentration of Arabidopsis increased by about 44% after 6 weeks treatment with 50 mM NaCl (Figure 3-19A, C). But this increase was not significant due to big standard errors (Table 3-6). No salt-induced change was found for the root S concentration in Arabidopsis (Figure 3-19B, D). The root S concentration of 10 week old control Thellungiella plants was higher than that of Arabidopsis plants (Figure 3-19B). During the 6 weeks treatment with 100 mM NaCl Thellungiella maintained constant S concentrations in both shoots and roots (Figure 3-19). The shoot/root ratio of the S concentration was higher in 10 week old control Arabidopsis plants than in the younger plants (Figure 3-17), whereas the shoot/root ratio of S in control Thellungiella plants did not change with age (Figure 3-17). Shoot/root ratios of S increased slightly after long-term salt treatment in both Arabidopsis and Thellungiella, from 6/10 to almost 9/10 in Arabidopsis, and from about 9/10 to 11/10 in Thellungiella (Figure 3-17B). However, neither of the changes was significant (Table 3-7).

3.3.4.3 Kinetics of changes in P and S concentrations during 72 h salt treatment

Changes in shoot P concentration of Arabidopsis and Thellungiella plants had similar kinetics to the decays of Ca and Mg concentrations. A transient decrease in the shoot P concentration occurred during the first 6 h of salt treatment (Figure 3-20A). Shoot P concentration decreased from 5.3 to 4.7 mg/g shoot DW in Arabidopsis plants, and from 4.3 to 4 mg/g shoot DW in Thellungiella. The shoot P concentration of Arabidopsis recovered after 24 h of salt application, then decreased again. After 72 h of salt treatment, the shoot P concentration of Arabidopsis plants was 4.5 mg/g shoot DW. Thellungiella shoots seemed to lack this second stage of P loss (Figure 3-20A). Interestingly, in the roots there was a transient increase of P during the initial 6 h of salt

treatment in both species (Figure 3-20B). After 6 h salt stress, the root P concentration increased from 6.8 to 8.3 mg/g root DW in Arabidopsis, and from 6.6 to 7.2 mg/g root DW in Thellungiella. After 24 h salt treatment, the root P concentrations dropped back to a level similar to the beginning of treatment and remained fairly constant in both species (Figure 3-20B).

A transient decrease during the first 6 h of salt treatment also occurred in the shoot S concentrations of Arabidopsis and Thellungiella plants (Figure 3-21A). It decreased from 3.9 to 3.4 mg/g shoot DW in Arabidopsis and from 7.4 to 6.8 mg/g shoot DW in Thellungiella, and recovered until 24 h after the application of salt treatment (Figure 3-21A). However, after 24 h of salt stress S concentrations of the shoots responded differently to the salt treatment. In Arabidopsis, a second phase of decrease occurred, whereas the shoot S concentration of Thellungiella continued to increase (Figure 3-21A). After salt treatment for 72 h, the shoot S concentration was 2.7 mg/g shoot DW in Arabidopsis, and 7.8 mg/g shoot DW in Thellungiella. In both species, the root S concentration increased transiently during the first 6 h of salt treatment, and then decreased quickly (Figure 3-21B). The decreases can be fitted with linear regression.

3.3.5 The effect of Ca and K supplements on tissue ion concentrations during short-term salt treatment and interaction between the effects of species, salt treatment and ion supplement

Figure 3-22 shows Na concentrations in the shoots (row a) and roots (row b) of Arabidopsis and Thellungiella grown with and without 25 h treatment with 100 mM NaCl (column 1) and additional 5 mM $CaCl_2$ (column 2) or 10 mM KCl (column 3). Figure 3-23 shows the means of root K (row a) and S (row b) concentrations, and Figure 3-24 shows root Ca (row a) and Mg (row b) concentrations of the two species before and after salt treatment and with or without Ca or K supplement. ANOVA was used to

determine not only whether the effects of species, salt treatment and ion supplement (Ca or K) on the respective ion concentrations were significant but also whether there were significant interactions between these effects (e.g. species and salt, ion-supplement and salt).

It has been previously shown that shoot Na concentrations differed between Arabidopsis and Thellungiella, and increased significantly after salt treatment in both species. ANOVA (Table 3-9) showed that the effects of species and salt treatment on shoot Na concentrations were not independent of each other, that is the increase in shoot Na concentration after salt treatment was higher in Arabidopsis than in Thellungiella ($p= 0.06$). The supplement of K or Ca did not significantly modulate the species and treatment dependent changes of shoot Na concentrations. In particular, the strong decrease in shoot Na by Ca determined for Arabidopsis (Figure 3-22(b)) was not significant due to large standard errors. Na concentrations of the roots were also different between the two species ('species', $p<0.0001$) and increased after salt treatment ('salt', $p<0.0001$). This increase was again significantly greater in Arabidopsis than in Thellungiella ('species' × 'salt', $p<0.0001$). For roots, both K and Ca supplement significantly decreased the Na concentration ('ion supplement', $p<0.0001$). The effects of K and Ca supplement on root Na concentrations were significantly stronger in Arabidopsis than in Thellungiella ('species' × 'ion supplement', $p=0.05$), and significantly stronger in salt treated plants than in control plants ('salt' × 'ion supplement', $p<0.0001$).

Shoot ion concentrations of the other ions measured were not altered by ion supplement, nor affected by any two inter-dependent factors (Table 3-9).

Root K concentrations were different between species and decreased during salt treatment ($p= 0.001$ for species and $p= 0.008$ for salt treatment). The effects of the two

factors were dependent on each other ('species' × 'salt', p= 0.007) i.e. the decrease was more pronounced in Arabidopsis than in Thellungiella. Supplements of Ca and surprisingly also of K, did not significantly change root K concentrations (Table 3-9).

Ca and Mg concentrations in the roots differed between the two species (p=0.013 for Ca, p=0.026 for Mg), and significantly decreased after salt treatment ('salt', p=0.007 for Ca, p<0.0001 for Mg). The root Ca concentration was increased by both K and Ca supplement ('ion supplement', p=0.006), whereas the root Mg concentration was decreased by supplementing with either of the two ions ('ion supplement', p=0.037). However, the ion supplements did not modulate the effects of species and salt on Ca or Mg concentrations.

ANOVA revealed that root S and P concentrations were significantly different between Arabidopsis and Thellungiella ('species', p<0.0001 for S, p=0.004 for P), and decreased after salt treatment ('salt', p<0.0001 for S, p=0.082 for P). For S this decrease occurred in Arabidopsis but not in Thellungiella ('species' × 'salt', p<0.0001). Interestingly, although K and Ca supplements did not significantly change root S concentrations due to large standard errors, there was a difference in the effects of ion supplements on root S concentration between the two species ('species' × 'ion supplement', p=0.087).

3.4 Discussion

Interesting results were obtained by comparing ion concentrations of Arabidopsis and Thellungiella plants after salt treatments for 25h and 6 weeks, studying the effects of additional Ca and K in the growth medium on this parameter, and determining the kinetics of net ion uptake/loss over a 72 h salt treatment. The species had different ion concentrations even under low salt conditions. Thellungiella plants contain considerably less divalent cations than Arabidopsis. Salt stress not only causes net accumulation of

Na in the plants, but also induces a net decrease in the concentration of other nutrient ions such as K, Ca, Mg, P and S, especially in Arabidopsis.

3.4.1 Difference in ion profiles between Arabidopsis and Thellungiella

Arabidopsis and Thellungiella plants have different ion compositions. Thellungiella plants have less Ca and Mg in the shoots and less K in the roots than Arabidopsis. In total, Thellungiella plants contain less than half as much inorganic cations as Arabidopsis plants. This implies a large difference in osmotic potential between the two species, unless Thellungiella plants contain other osmotica such as organic solutes to compensate for the difference in inorganic ions. Previous studies have found that the proline content was higher in Thellungiella plants than in Arabidopsis plants (Gong et al., 2005; Inan et al., 2004; Taji et al., 2004a), and microarray analysis in this project revealed that Thellungiella plants express more P5CS (Delta1-pyrroline-5-carboxylate synthase) than Arabidopsis both in low and high salt conditions (see Chapter 4).

3.4.2 Loss of K

Other than Na accumulation, the most significant salt-induced change in tissue ion concentrations was a decrease of K concentration in both roots and shoots of Arabidopsis. Considering that there is no significant growth of the plants during the 25 h treatment period, the decrease in K concentration is not a dilution effect of growth but reflects actual net loss of K from the plants. The K loss from the roots of Arabidopsis occurred at a rate of 0.3 mg/g root DW. Hardly any K was lost from Thellungiella roots over the same period of time (0.1 mg/g root DW). Surprisingly, the K concentration in the roots of Arabidopsis transiently increased in the six hours immediately after salt application (Figure 3-5B). However, the K concentration determined at time point 0 was considerably lower than the one determined in the previous experiment (Figure 3-2B), therefore it must be assumed that there is an experimental error in this value in the time

course experiment. The source of this error could not be identified. The simplest explanation for the observed K loss in Arabidopsis is that the rise in the extracellular Na concentration causes a depolarisation which in turn causes K efflux through outward-rectifying K channels. Indeed, it was found that addition of NaCl to the external medium causes a large depolarisation of Arabidopsis root cells (Volkov and Amtmann, unpublished results). Root cells of Thellungiella in the same conditions showed a much smaller depolarisation. Loss of K from the shoots started later than in the roots (after 24 h of salt treatment) and occurred at a lower rate, which was similar in both species. This is astonishing since Arabidopsis shoot cells experience higher Na levels than Thellungiella shoot cells. It could be re-absorbed whereas in the roots apoplastic K is quickly lost from the plants. The transient increase in shoot K concentration was also found in both species. The reason for this is unclear.

After long-term (6 weeks) salt treatment, both species had lower K concentrations in roots and shoots than under control conditions. However, at least in Arabidopsis, K concentrations were higher than expected from the initial rate of K loss. It shows that during long-term salt stress plants have mechanisms to control K fluxes so that they achieve a new equilibrium. One mechanism is to induce measures that re-polarise the membrane. This would include activating the H pump and closing Na permeable channels. Indeed, increased transcript levels of the plasma membrane proton pump AHA2 were found in Arabidopsis roots after salt treatment (Maathuis et al., 2003). Furthermore, it has been reported that salt stress evokes a cytoplasmic cGMP signal in Arabidopsis (Donaldson et al., 2004). Elevated cytoplasmic levels of cGMP inhibit the activity of a voltage-independent non-selective channel in Arabidopsis that appear to be the main pathway for Na uptake (Maathuis and Sanders, 2001).

3.4.3 Changes in other cations

Salt treatment induced not only loss of K but also the loss of other cation ions such as Ca and Mg. Loss of Ca and Mg from the roots was fitted with a single or double exponential decay model with time constants of 0.18 h for Arabidopsis Ca, 285 h for Thellungiella Ca, 4 h for Arabidopsis Mg and 6.67 h for Thellungiella Mg.

Rate of divalent cation loss (especially in the case of Ca) suggests that it reflects at least in part replacement of cell wall bound divalent cations by Na. Release of intracellular Ca and Mg could again be the result of membrane depolarisation. However, the membrane potential is unlikely to reach values positive of E_{Ca} and E_{Mg}. Therefore net loss of Ca and Mg is not due to unidirectional efflux (as in the case of K), but to a decrease in Ca and Mg uptake. Thus, a net efflux of these two ions will occur as long as their unidirectional efflux is maintained.

Very little divalent cations are released from the shoots of both species and the overall difference in Ca and Mg shoot contents between the two species persists during salt stress although Arabidopsis accumulates more Na. This indicates that Ca and Mg form stable complexes in the vacuole (e.g. as Ca-oxalate, Li et al., 2003) and are no longer available for ion homeostasis (Li et al., 2003).

3.4.4 Changes in anion concentrations

Thellungiella plants contain slightly less P and more S than Arabidopsis plants. P and S contents decreased in both roots and shoots of Arabidopsis after short-term salt treatment, whereas in Thellungiella they were not affected by short-term salt stress. However P and S contents in the shoots of Arabidopsis increased after long-term salt treatment. Therefore plants might have mechanisms to adjust the expression and/or activity of P and S transporter genes during salt stress. Such regulation was indeed apparent in the microarray analysis particularly for Thellungiella (see Chapter 4). It can

be concluded that although Arabidopsis does maintain its capacity to take up P and S under moderate long-term salt stress (50 mM NaCl), it is less capable than Thellungiella to do so during short-term treatment with higher NaCl concentration (100 mM). Unfortunately this study did not include measurements of Cl since this anion cannot be measured with ICP-OES. However, energy-dispersive X-ray analysis of leaf cells showed that both species had accumulated large amounts of Cl after long-term treatment with NaCl (Volkov et al., 2004). It appears that this increase in Cl levels was not compensated by a loss of other anions (such as P and S) but neutralized by the parallel uptake of Na.

3.4.5 Ion tissue allocation

Arabidopsis plants preferentially store divalent cations such as Ca and Mg in the shoots. Shoot/root ratios of these ions in Arabidopsis are at least double the shoot/root ratios in Thellungiella. During salt stress, Arabidopsis plants maintain the high shoot/root ratios or even over-accumulate divalent cations in the shoots, whereas in Thellungiella shoot/root ratios of Ca and Mg are much more flexible. Thellungiella plants equally distribute K ions between the shoots and roots in the early growth stage. Interestingly the only other element that is evenly distributed in Thellungiella plants is S. This coincidence suggests that K and SO_4 long distance movement is coupled. However when the plants grow older, K was preferentially stored in the roots of Thellungiella plants whereas S distribution did not change during plant development. Arabidopsis showed a K distribution pattern that was the opposite of Thellungiella. In younger plants K is preferentially allocated in the roots, but the shoot/root ratio of K increases when the plants grow older. The distribution of S in Arabidopsis plants also resembles the distribution of K. As in the case of Ca and Mg Arabidopsis plants preferentially accumulate K and S in the shoots during short-term salt stress. It appears that the

glycophytic and the halophytic species fundamentally differ in their K allocation pattern. Whether this difference is functionally linked to the difference in salt tolerance remains to be studied. Interestingly, it was observed that after several months of growth in high salt medium Thellungiella plants developed simple salt glands on their leaves. These glands did not export Na but K salt (Volkov, unpublished results).

The distribution of P is similar in Arabidopsis and Thellungiella. Both plants preferentially accumulate P in the roots, but the shoot/root ratio of P content in Thellungiella plants decreases as the plants grow older. Again, Arabidopsis plants preferentially accumulate P in the shoots during salt stress.

3.4.6 Effect of Ca and K supplements on Na concentrations

It is well known that increased levels of Ca and K in the growth medium improve growth of salt stressed plants (Lahaye and Epstein, 1969; Lauchli, 1990b). This effect might have several explanations, including improved stability of the plasma membrane by Ca, increased cytoplasmic K/Na ratios and inhibition of Na uptake. In this study it was shown that increasing external Ca and K concentrations from 0.5 mM to 5 mM and from 2 mM to 10 mM respectively had significant effects on root Na concentrations in both species. Na concentrations were decreased by both ion supplements and this effect was stronger in salt-treated plants than in control plants. The results clearly show that the ameliorating effect of Ca and K in salt-stressed plants is at least partly due to their interference with Na transport. This means that in both species Na uptake has a component that can be inhibited by Ca and K. For Ca, the decrease in root Ca concentration over 24 h can be compared with the observed inhibition of unidirectional Na uptake by external Ca. Although the K_i of this effect is in the μM range, increasing external Ca from 0.5 to 5 mM will still decreases Na uptake in 15 m by 40% in Thellungiella (from 2.2 to 1.3 μmol, Figure 2-13). This number agrees with the

difference in root Na concentrations observed in 0.5 mM and 5 mM Ca (Figure 3-22). For Arabidopsis, Ca inhibition of unidirectional Na influx was measured by Essah et al. (2003). It is about 50% inhibition in 50 mM NaCl which is comparable to the inhibition effect in Thellungiella. The ANOVA indicated a stronger inhibitory effect by increased external Ca concentration on root Na concentrations in Arabidopsis than in Thellungiella (Figure 3-22b). Ca inhibition of unidirectional Na uptake can be ascribed to Ca-sensitivity of voltage-independent channels, which was confirmed for both Arabidopsis (Demidchik and Tester, 2002) and Thellungiella (Volkov and Amtmann, submitted). Although quantitative comparison between the two species is difficult due to different experimental conditions, it appears that Ca-dependence of voltage-independent channels is similar in the two species. However, the finding that this pathway conducts more Na in Arabidopsis than in Thellungiella (Volkov et al., 2004; Volkov and Amtmann, submitted) provides a reasonable explanation for the larger effect of Ca supplementation on Na accumulation in Arabidopsis than in Thellungiella.

The observed effect of K supplementation on root Na concentration is more difficult to interpret particularly as additional K did not increase the K concentration in the roots (Figure 3-23a3). Competitive inhibition of Na uptake by K (e.g. in HKT1-type or KUP/HAK type transporters) would lead to increased K concentrations unless K efflux is increased simultaneously. However, K might inhibit Na uptake pathways without permeating. For example, inhibition of K uptake by Na but not *vice versa* has been observed for HKT1 (Rubio et al., 1999). Effects of external K on the activity (gating) of K selective inward and outward rectifying channels are well known (Blatt, 1992; Schachtman, 2000). The possibility that external K also regulates voltage-independent channels has not been investigated and would require further electrophysiological studies.

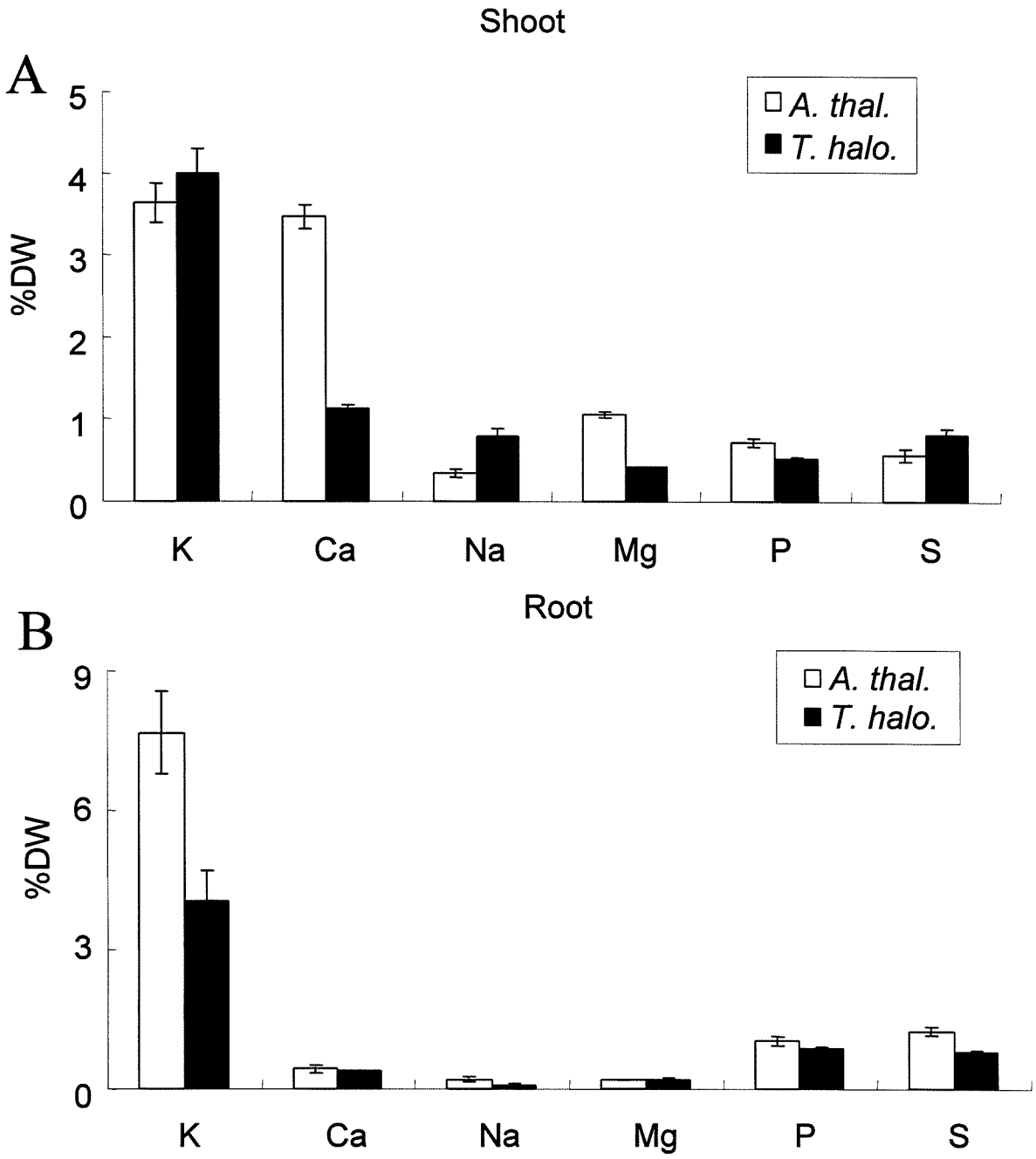

Figure 3-1. Ion profile of 4 week old Arabidopsis (white bars) and Thellungiella (black bars) plants growing in a minimum nutrient solution. Six to seven plants were pooled for each replicate. These figures present absolute values as percent of dry weight in the shoots (A) and the roots (B). Values are the mean ± SE (n=4).

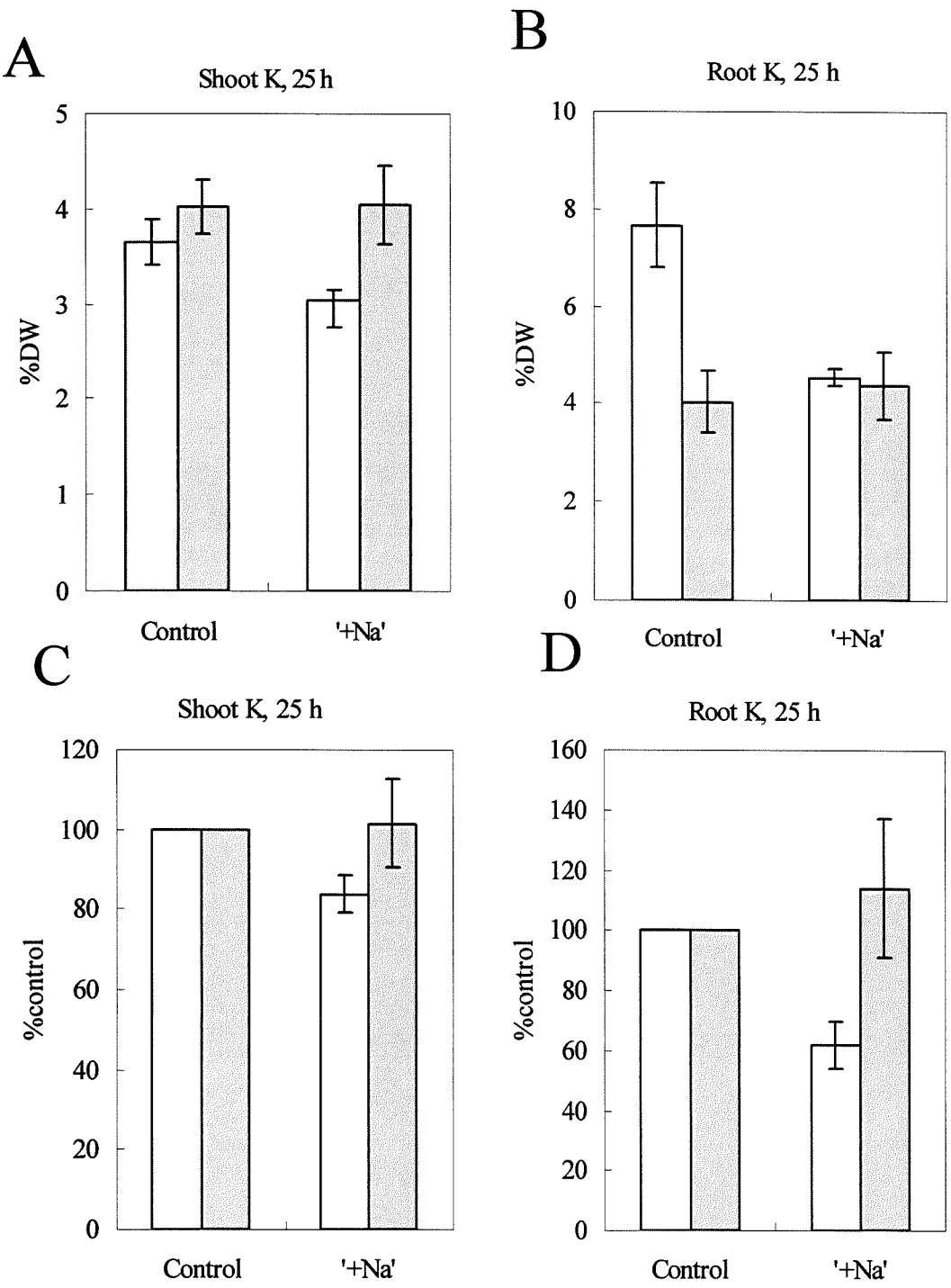

Figure 3-2. Potassium concentrations of Arabidopsis (white bars) and Thellungiella (grey bars) plants after 25 h exposure to 'control' or '+Na' (100 mM NaCl) medium. Six to seven plants were pooled for each replicate. These figures present absolute values as percent of dry weight in shoot (A) and root (B), and relative changes to the control level within each experiments in shoot (C) and root (D). Values are the mean ± SE (n=4).

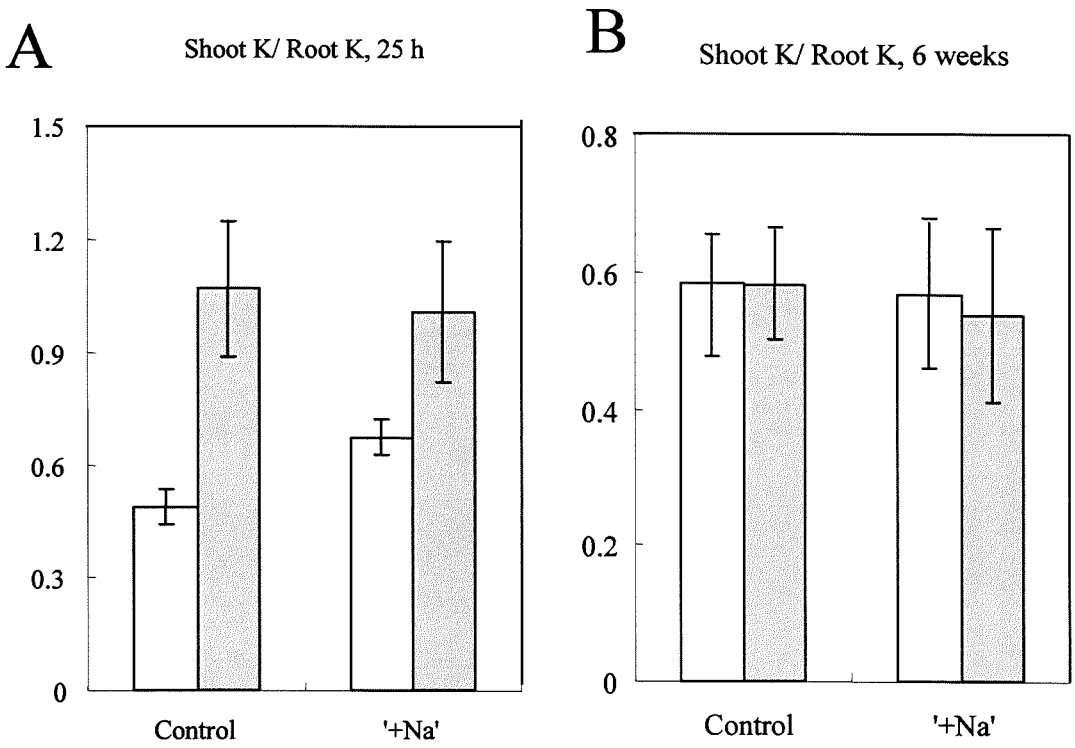

Figure 3-3. Shoot/ root ratios of K concentration in Arabidopsis (white bars) and Thellungiella (grey bars) plants with and without short- (A) and long- (B) term salt treatment. The short-term (25 h) salt treatment consisted of growth medium with 100 mM NaCl. The long-term (6 weeks) salt treatments consisted of 50 mM NaCl for Arabidopsis and 100 mM NaCl for Thellungiella respectively. Six to seven plants were pooled for each replicate. Values are the mean ± SE (n=4).

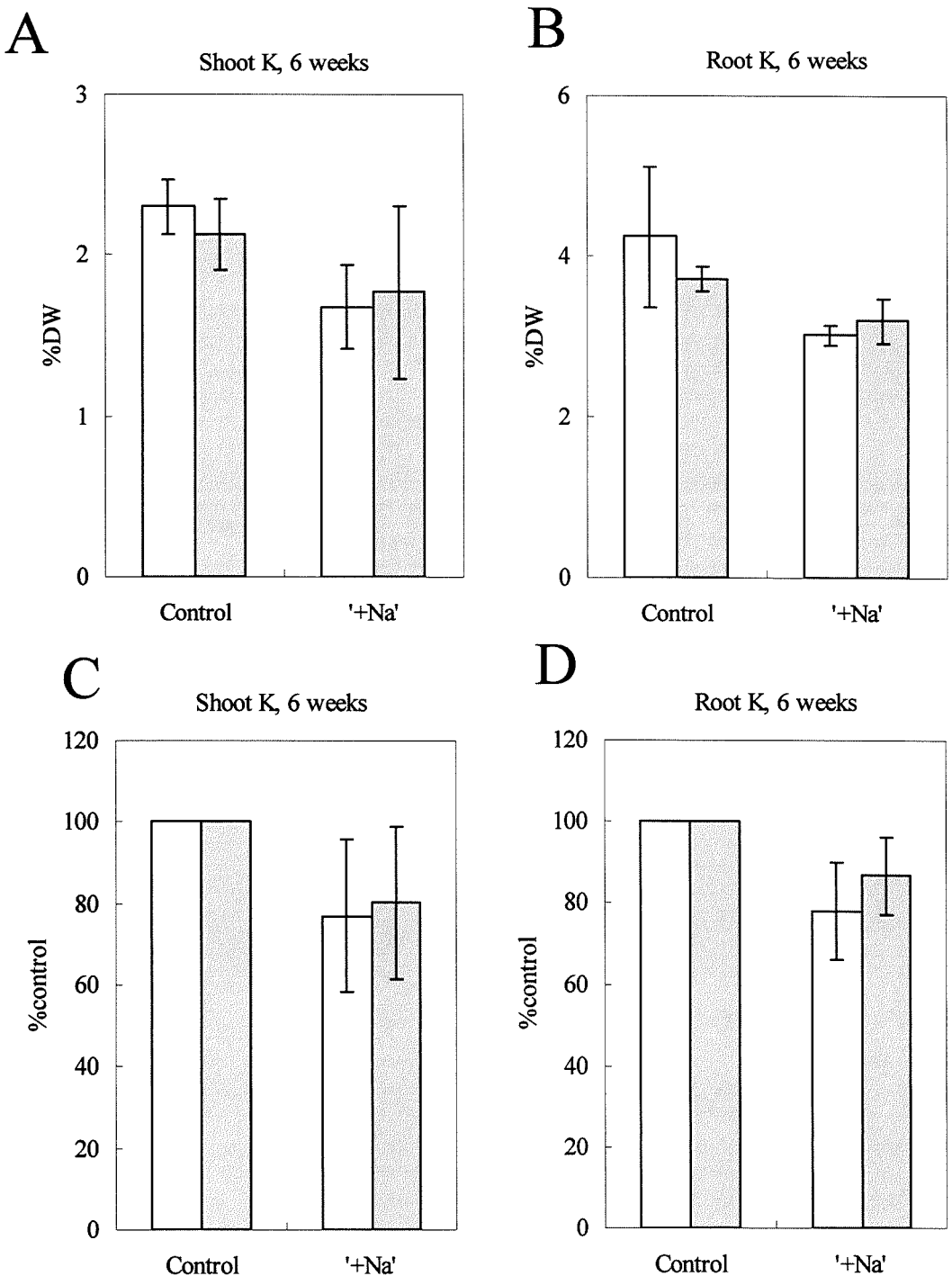

Figure 3-4. Potassium concentrations of Arabidopsis (white bars) and Thellungiella (grey bars) plants after 6 weeks exposure to 'control' or '+Na' (At: 50 mM NaCl, Th: 100 mM NaCl) medium. Six to seven plants were pooled for each replicate. These figures present absolute values as percent of dry weight in shoot (A) and root (B), and relative changes to the control level within each experiment in shoot (C) and root (D). Values are the mean ± SE (n=4).

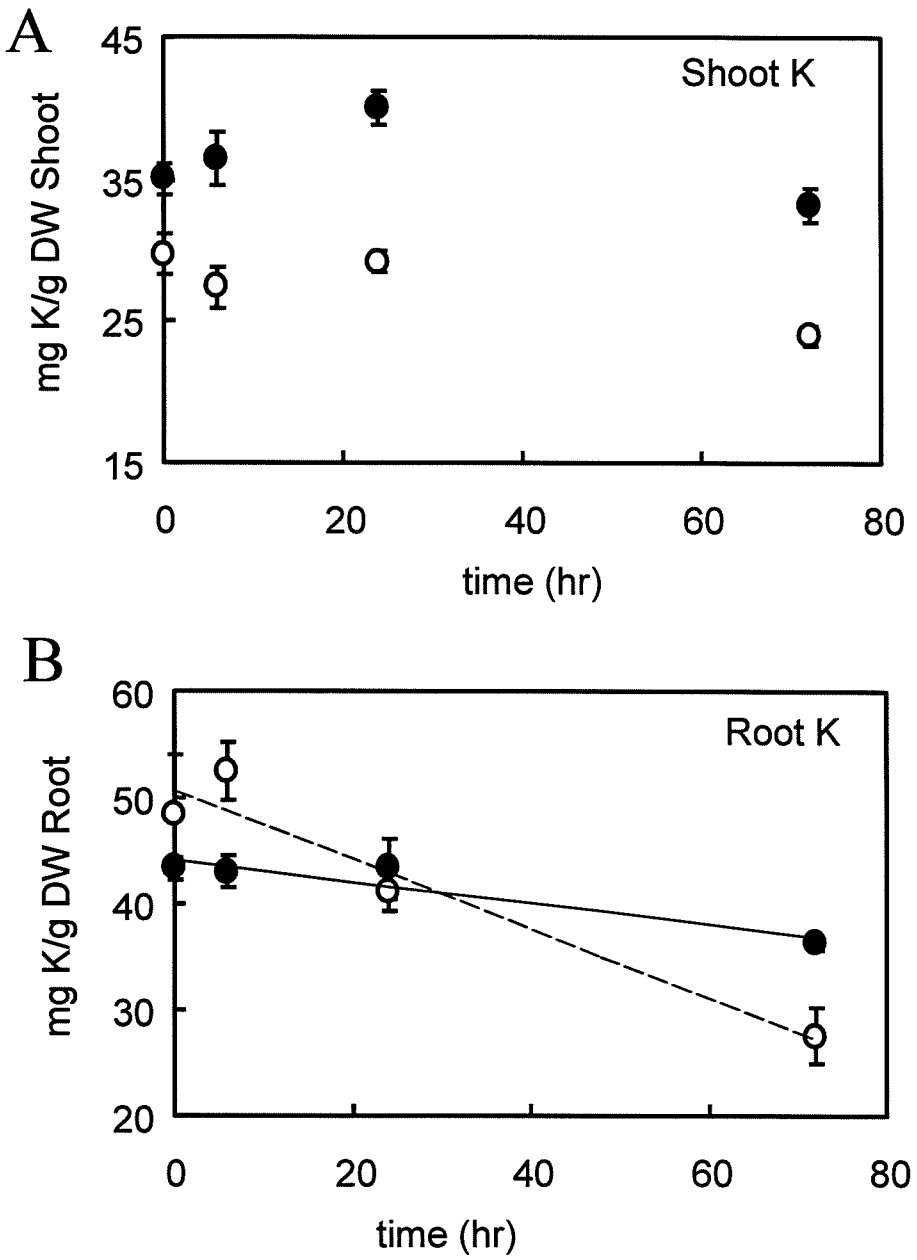

Figure 3-5. Kinetics of the changes in K concentrations in the shoots (A) and roots (B) of Arabidopsis (open circles) and Thellungiella (closed circles) plants during a 72 h treatment with 100 mM NaCl in hydroponic growth solution. Ion concentrations of single plants were determined by ICP-OES. Values are the mean ± SE (n=9).

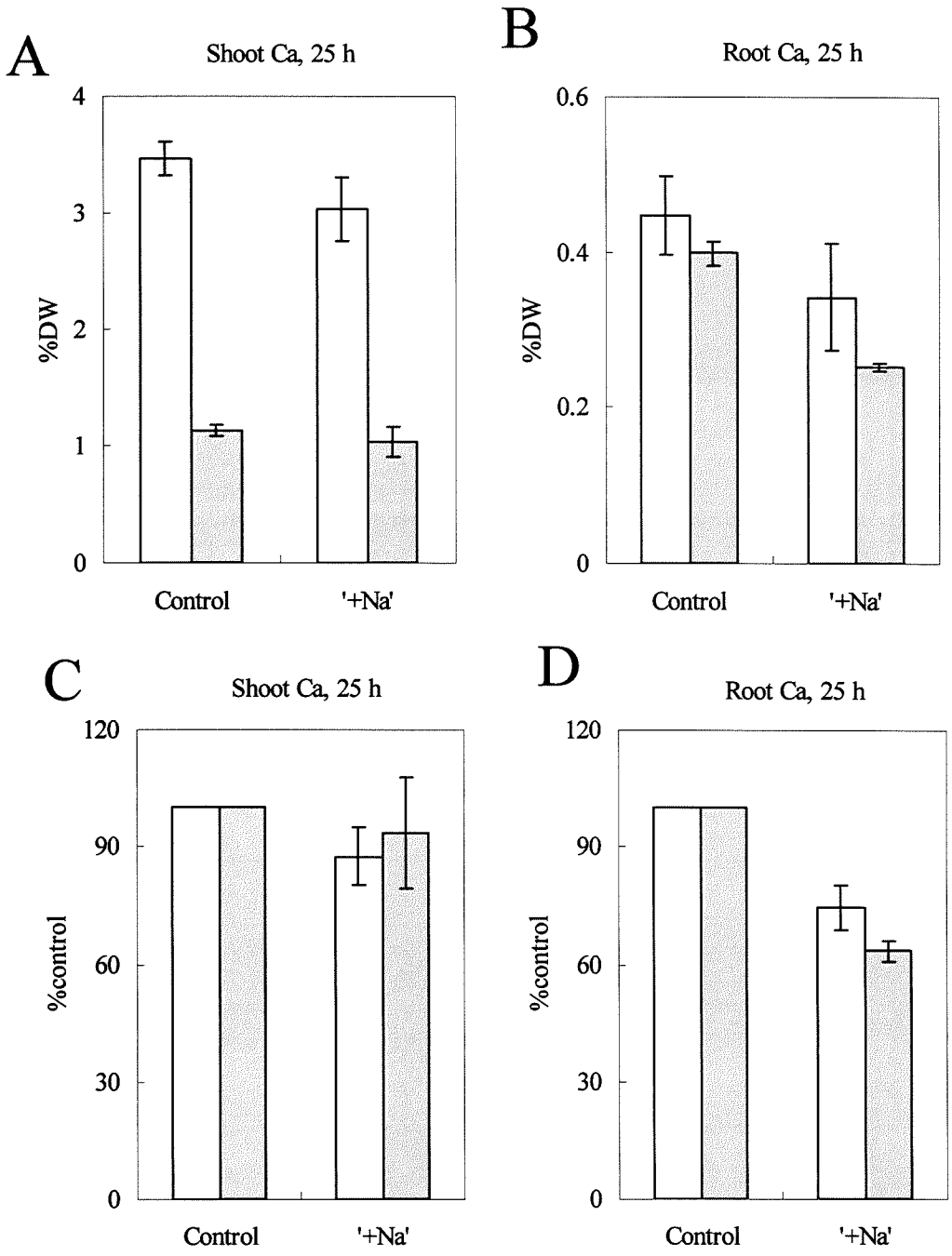

Figure 3-6. Calcium concentrations of Arabidopsis (white bars) and Thellungiella (grey bars) plants after 25 h exposure to 'control' or '+Na' (100 mM NaCl) medium. Six to seven plants were pooled for each replicate. These figures present absolute values as percent of dry weight in shoot (A) and root (B), and relative changes to the control level within each experiment in shoot (C) and root (D). Values are the mean ± SE (n=4).

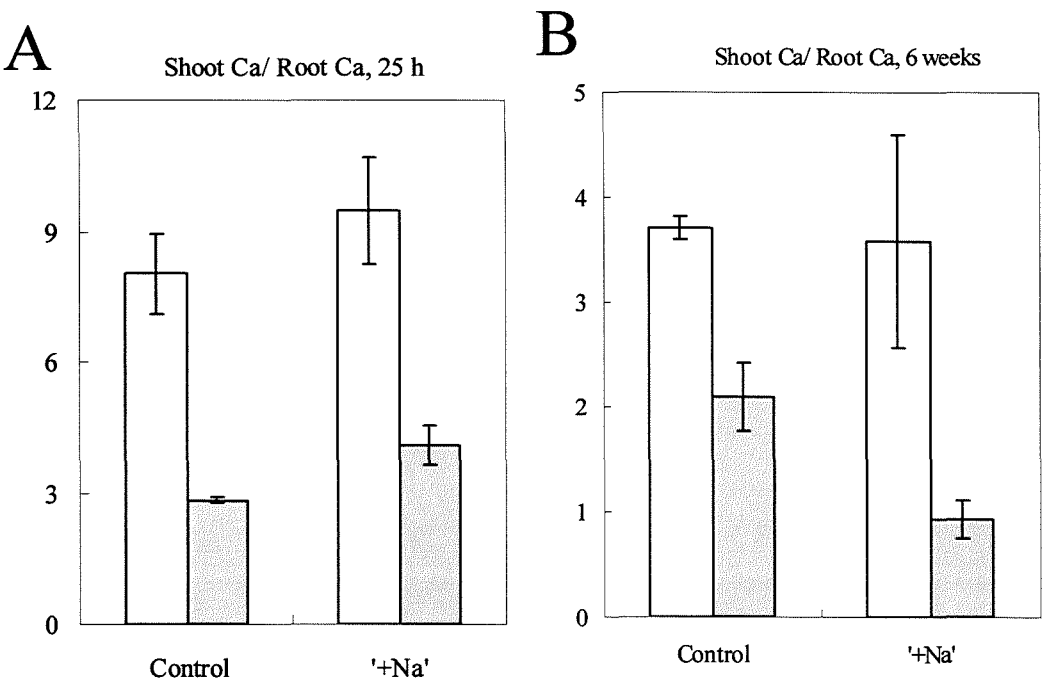

Figure 3-7. Shoot/ root ratios of Ca concentration in Arabidopsis (white bars) and Thellungiella (grey bars) plants with and without short- (A) and long- (B) term salt treatment. The short-term (25 h) salt treatment consisted of 100 mM NaCl growth medium. The long-term (6 weeks) salt treatments consisted of 50 mM NaCl for Arabidopsis and 100 mM NaCl for Thellungiella respectively. Six to seven plants were pooled for each replicate. Values are the mean ± SE (n=4).

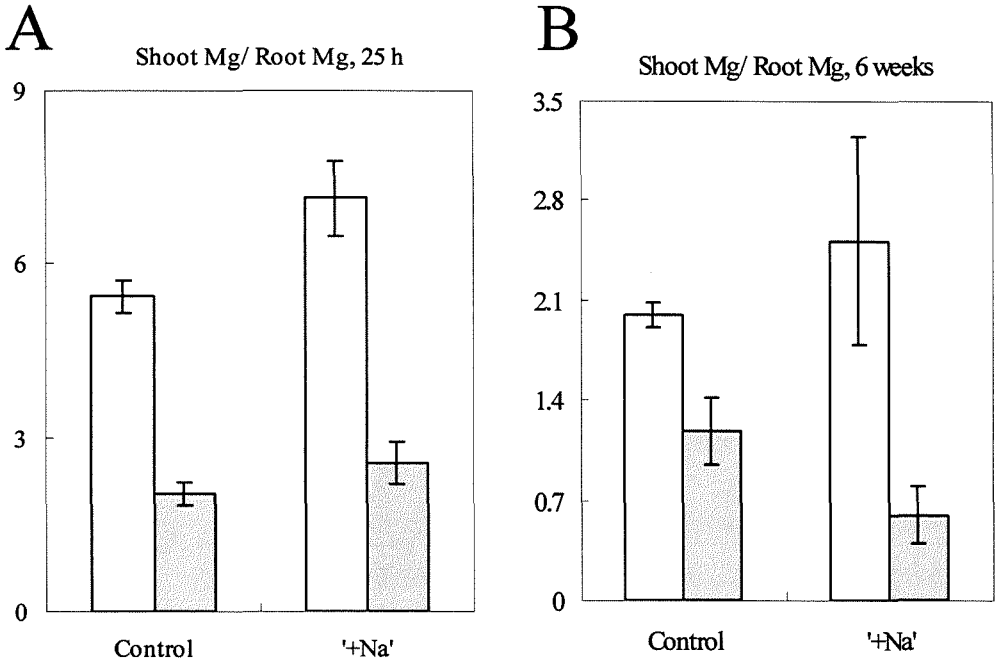

Figure 3-8. Shoot/ root ratios of Mg concentration in Arabidopsis (white bars) and Thellungiella (grey bars) plants with and without short- (A) and long- (B) term salt treatment. The short-term (25 h) salt treatment consisted of 100 mM NaCl. The long-term (6 weeks) salt treatments consisted of 50 mM NaCl for Arabidopsis and 100 mM NaCl for Thellungiella respectively. Six to seven plants were pooled for each replicate. Values are the mean ± SE (n=4).

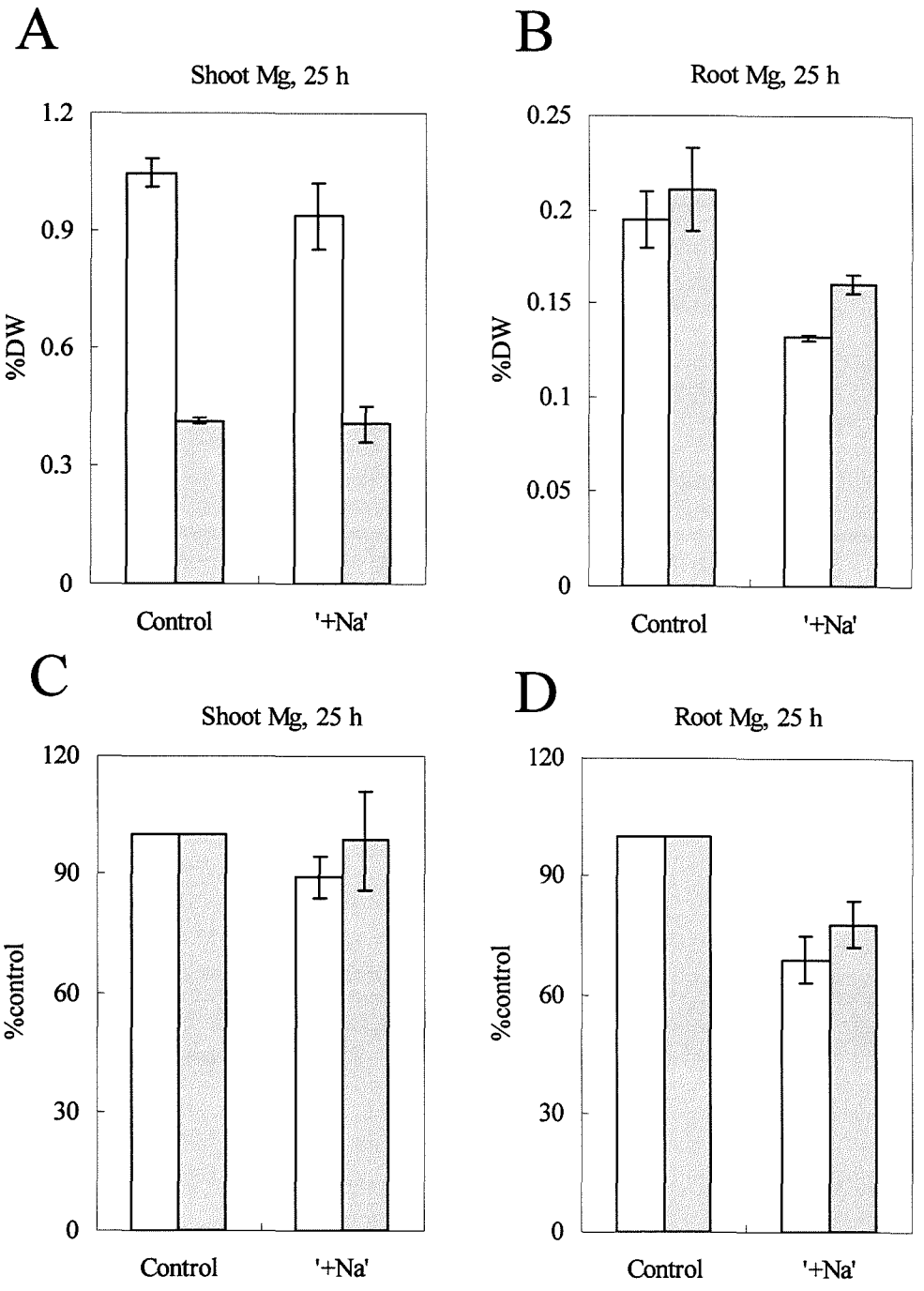

Figure 3-9. Magnesium concentrations of Arabidopsis (white bars) and Thellungiella (grey bars) plants after 25 h exposure to 'control' or '+Na' (100 mM NaCl) medium. Six to seven plants were pooled for each replicate. These figures present absolute values as percent of dry weight in shoot (A) and root (B), and relative changes to the control level within each experiments in shoot (C) and root (D). Values are the mean ± SE (n=4).

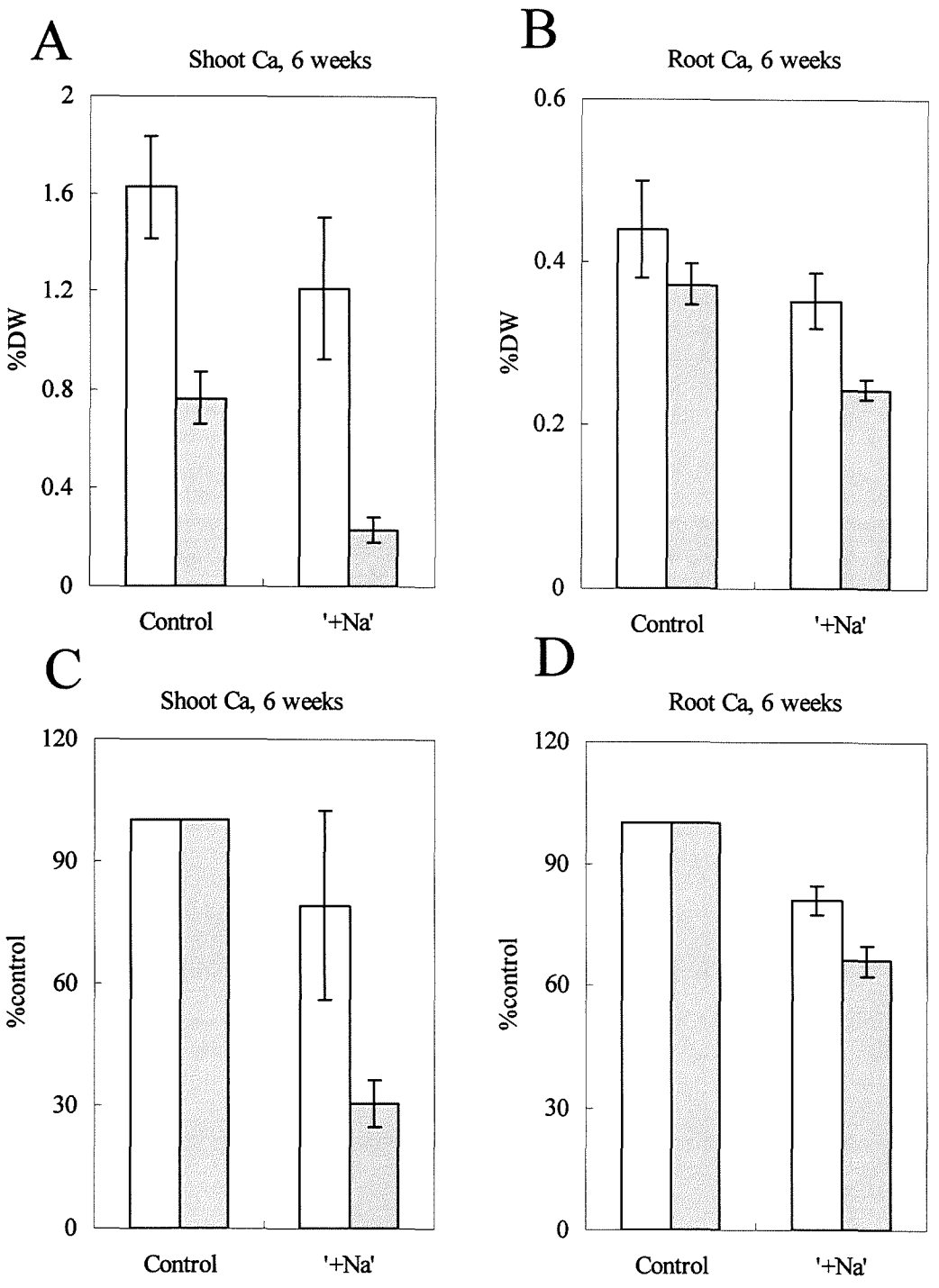

Figure 3-10. Calcium concentrations of Arabidopsis (white bars) and Thellungiella (grey bars) plants after 6 weeks exposure to 'control' or '+Na' (At: 50 mM NaCl, Th: 100 mM NaCl) medium. Six to seven plants were pooled for each replicate. These figures present absolute values as percent of dry weight in shoot (A) and root (B), and relative changes to the control level within each experiment in shoot (C) and root (D). Values are the mean ± SE (n=4).

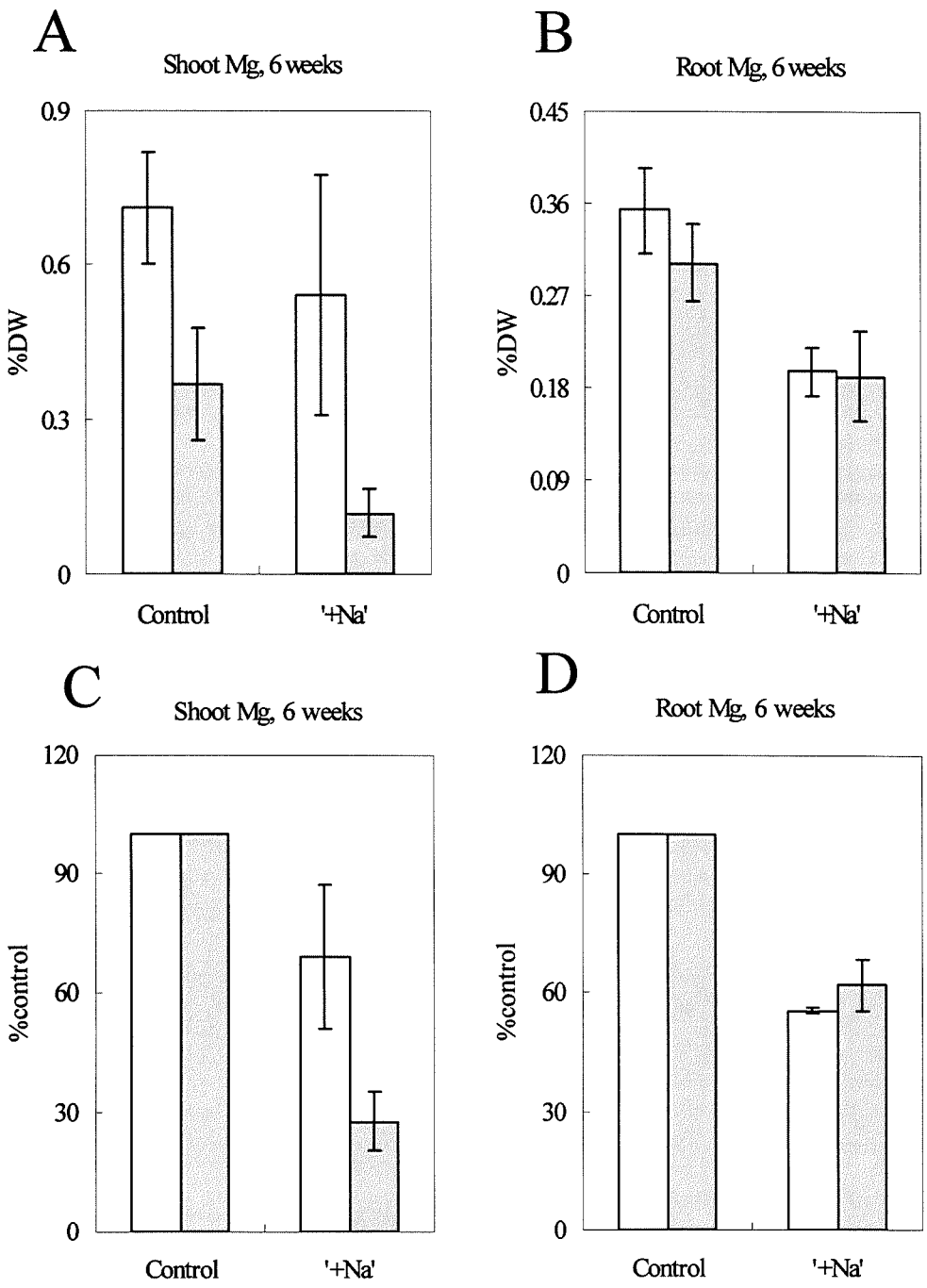

Figure 3-11. Magnesium concentrations of Arabidopsis (white bars) and Thellungiella (grey bars) plants after 6 weeks exposure to 'control' or '+Na' (At: 50 mM NaCl, Th: 100 mM NaCl) medium. Six to seven plants were pooled for each replicate. These figures present absolute values as percent of dry weight in shoot (A) and root (B), and relative changes to the control level within each experiment in shoot (C) and root (D). Values are the mean ± SE (n=4).

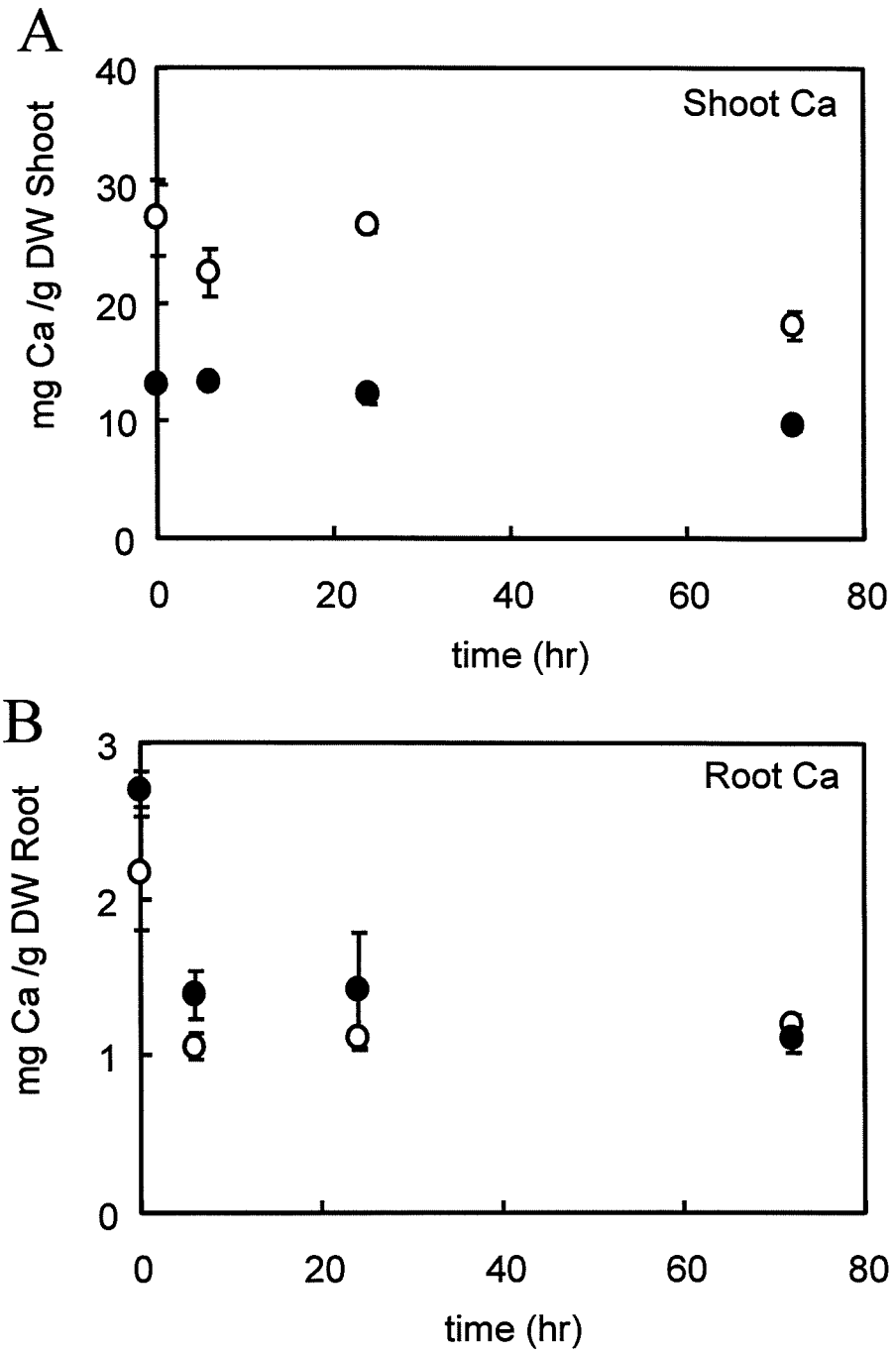

Figure 3-12. Kinetics of the changes in Ca concentration in the shoots (A) and roots (B) of Arabidopsis (open circles) and Thellungiella (closed circles) plants during a 72 h treatment with 100 mM NaCl in hydroponic growth solution. Ion concentrations of single plants were determined by ICP-OES. Values are the mean ± SE (n=9).

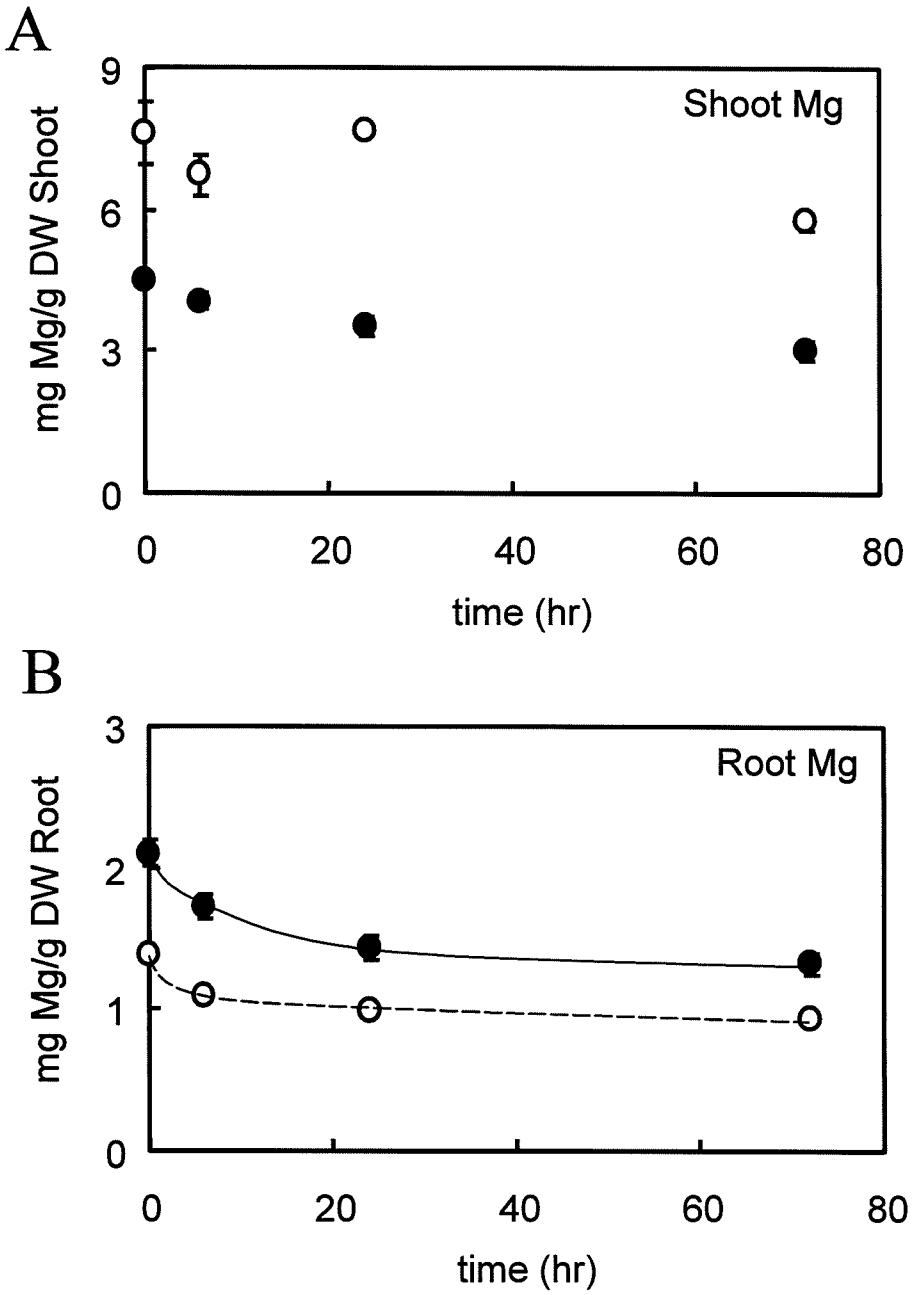

Figure 3-13. Kinetics of the changes in Mg concentration in the shoots (A) and roots (B) of Arabidopsis (open circles) and Thellungiella (closed circles) plants during a 72 h treatment with 100 mM NaCl in hydroponic growth solution. Ion concentrations of single plants were determined by ICP-OES. Values are the mean ± SE (n=9).

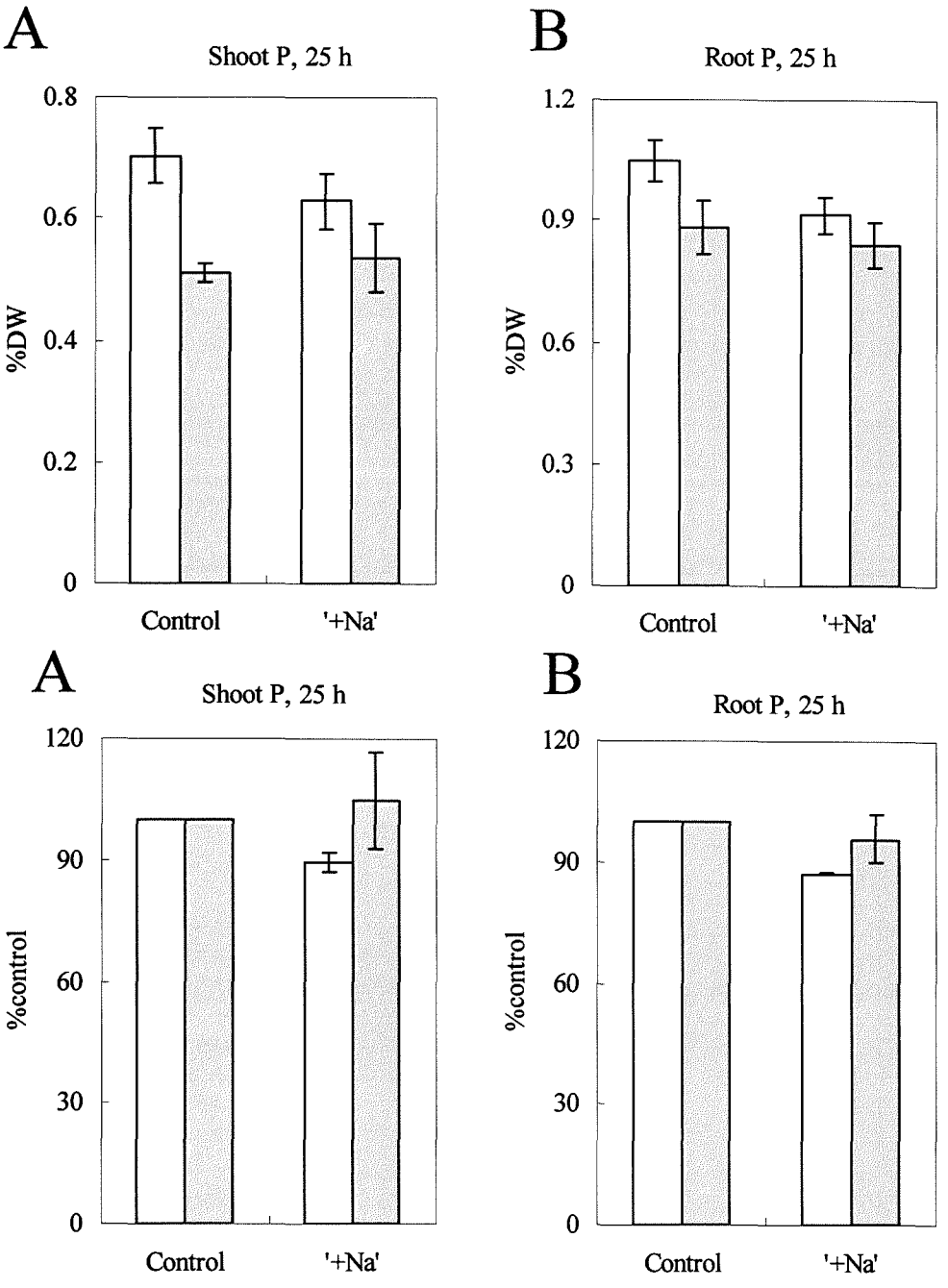

Figure 3-14. Phosphorus concentration of Arabidopsis (white bars) and Thellungiella (grey bars) plants after 25 h exposure to 'control' or '+Na' (100 mM NaCl) medium. Six to seven plants were pooled for each replicate. These figures present absolute values as percent of dry weight in shoot (A) and root (B), and relative changes to the control level within each experiments in shoot (C) and root (D). Values are the mean ± SE (n=4).

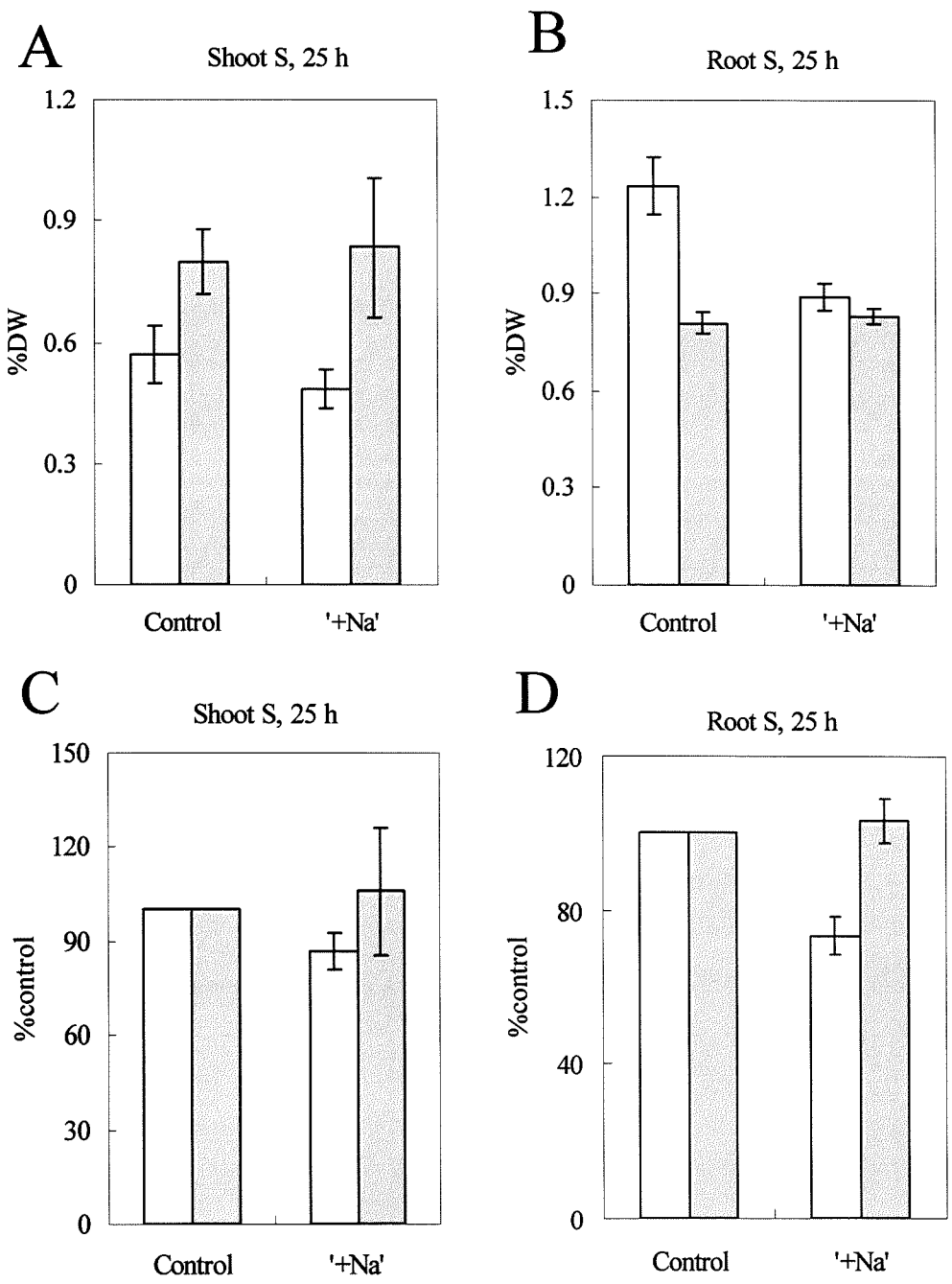

Figure 3-15. Sulfur concentration of Arabidopsis (white bars) and Thellungiella (grey bars) plants after 25 h exposure to 'control' or '+Na' (100 mM NaCl) medium. Six to seven plants were pooled for each replicate. Averages of four independent experiments are shown. Error bars are SE. These figures present absolute values as percent of dry weight in shoot (A) and root (B), and relative changes to the control level within each experiments in shoot (C) and root (D). Values are the mean ± SE (n=4).

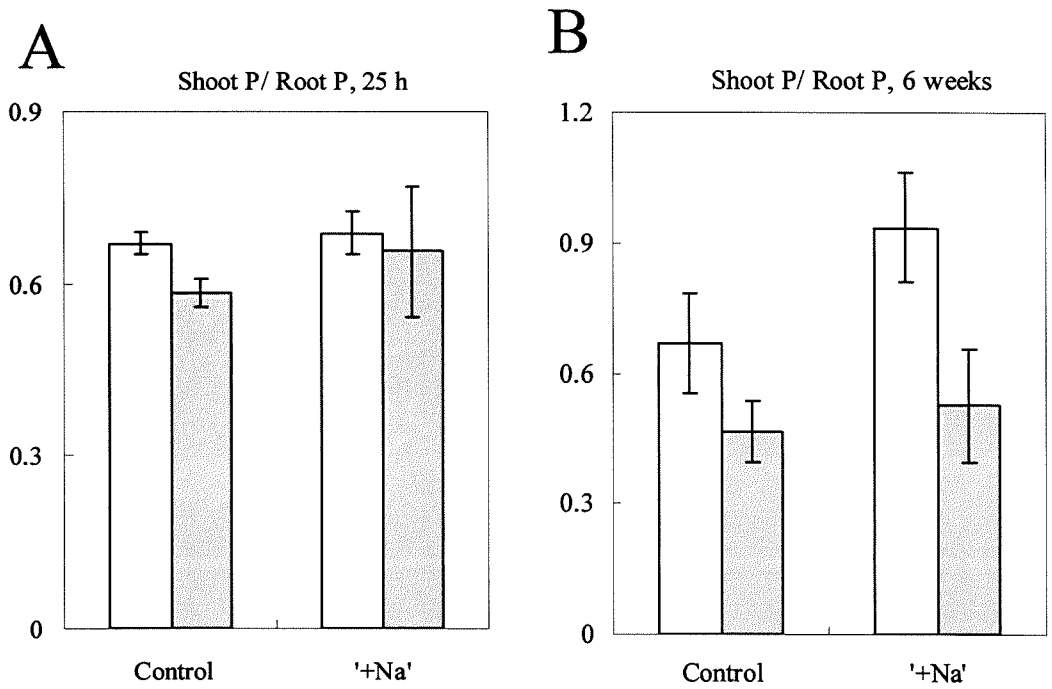

Figure 3-16. Shoot/ root ratios of P concentration in Arabidopsis (white bars) and Thellungiella (grey bars) plants with and without short- (A) and long- (B) term salt treatment. The short-term (25 h) salt treatment consisted of 100 mM NaCl. The long-term (6 weeks) salt treatments consisted of 50 mM NaCl for Arabidopsis and 100 mM NaCl for Thellungiella respectively. Six to seven plants were pooled for each replicate. Values are the mean ± SE (n=4).

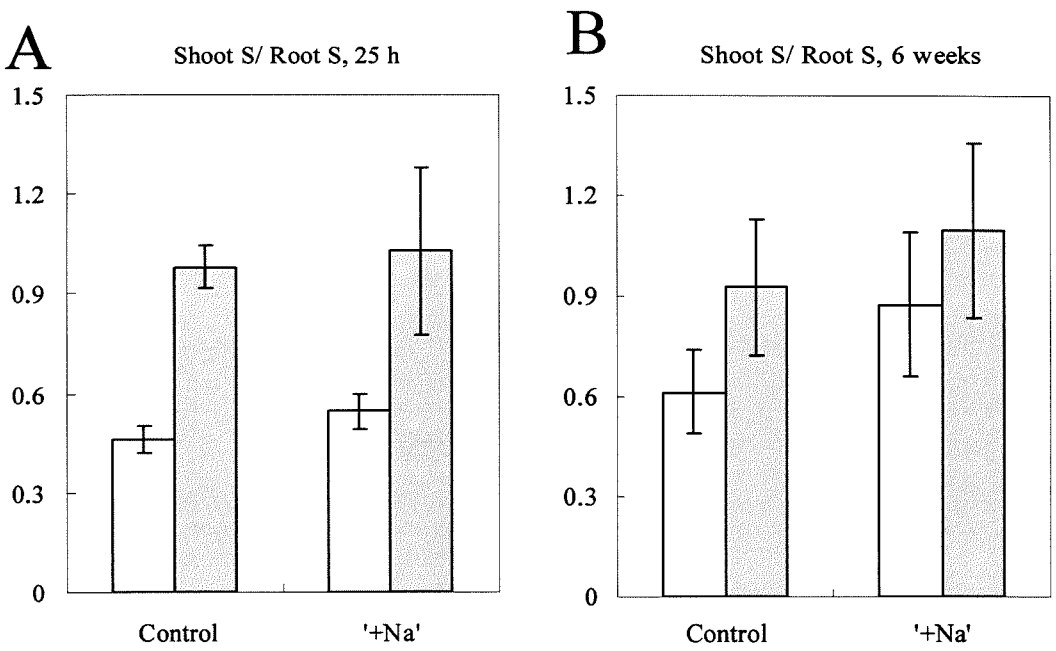

Figure 3-17. Shoot/ root ratios of S concentration in Arabidopsis (white bars) and Thellungiella (grey bars) plants with and without short- (A) and long- (B) term salt treatment. The short-term (25 h) salt treatment consisted of 100 mM NaCl. The long-term (6 weeks) salt treatments consisted of 50 mM NaCl for Arabidopsis and 100 mM NaCl for Thellungiella respectively. Six to seven plants were pooled for each replicate. Values are the mean ± SE (n=4).

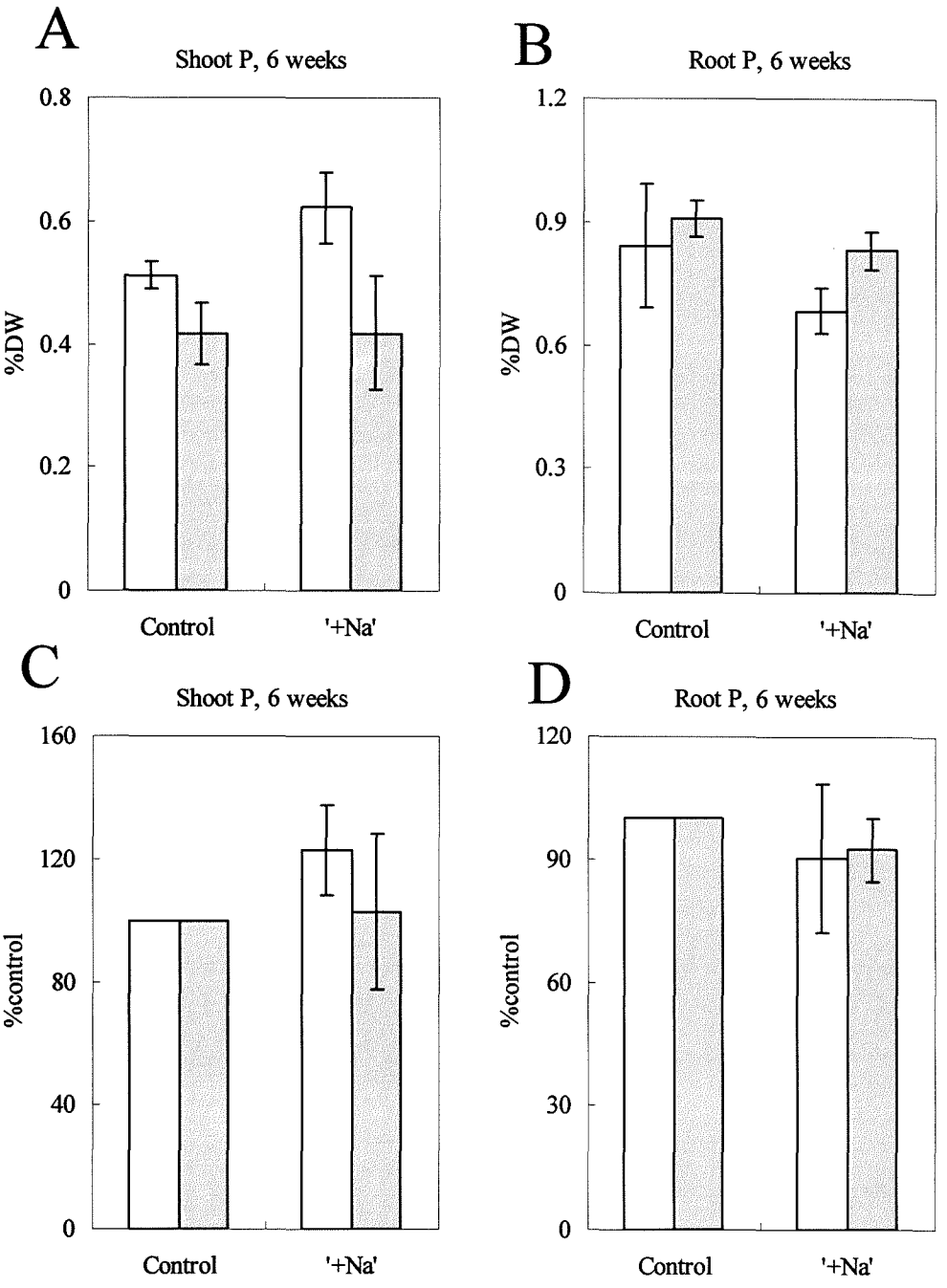

Figure 3-18. Phosphorus concentration of Arabidopsis (white bars) and Thellungiella (grey bars) plants after 6 weeks exposure to 'control' or '+Na' (At: 50 mM NaCl, Th: 100 mM NaCl) medium. Six to seven plants were pooled for each replicate. These figures present absolute values as percent of dry weight in shoot (A) and root (B), and relative changes to the control level within each experiment in shoot (C) and root (D). Values are the mean ± SE (n=4).

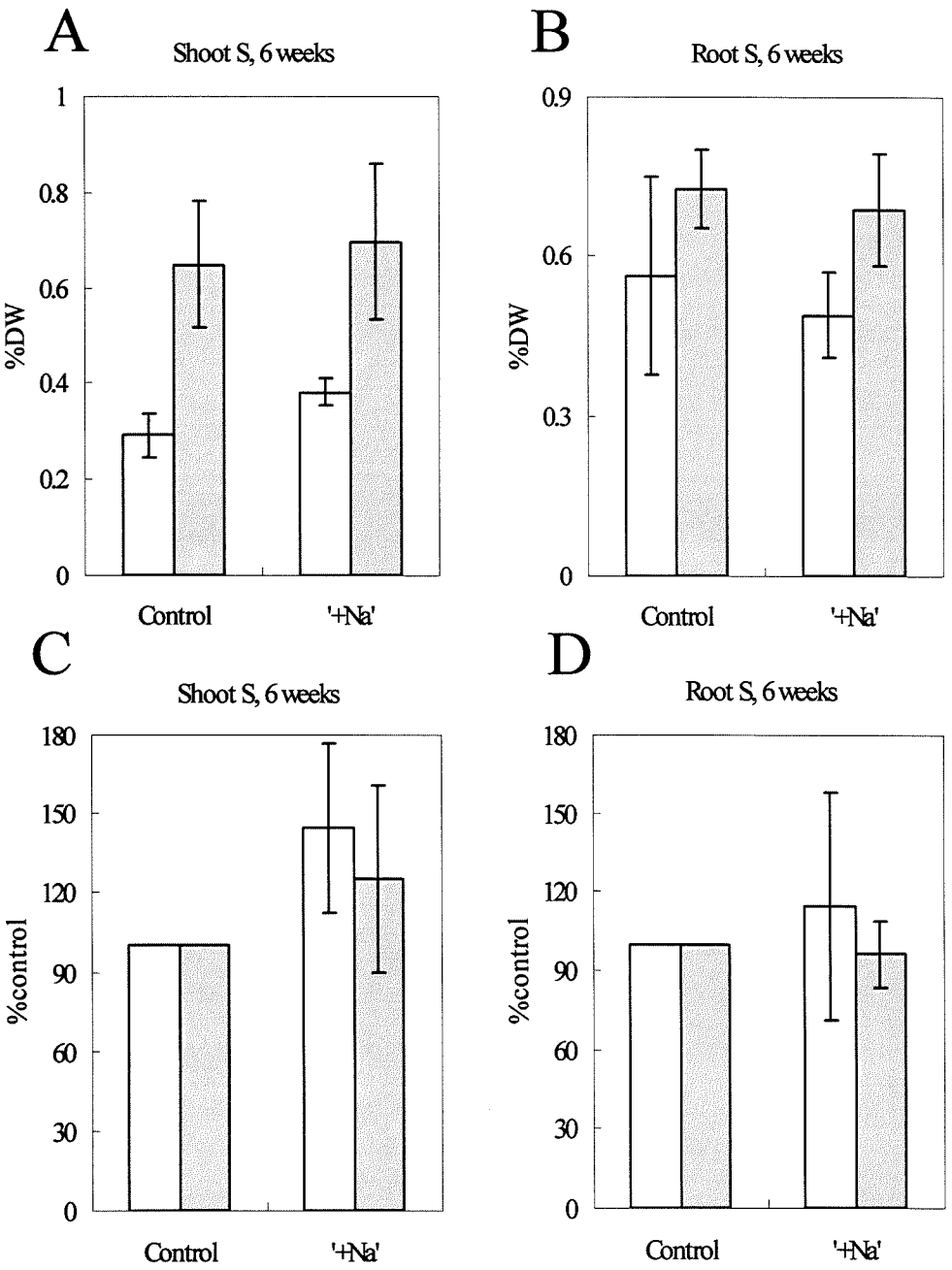

Figure 3-19. Sulfur concentration of Arabidopsis (white bars) and Thellungiella (grey bars) plants after 6 weeks exposure to 'control' or '+Na' (At: 50 mM NaCl, Th: 100 mM NaCl) medium. Six to seven plants were pooled for each replicate. These figures present absolute values as percent of dry weight in shoot (A) and root (B), and relative changes to the control level within each experiment in shoot (C) and root (D). Values are the mean ± SE (n=4).

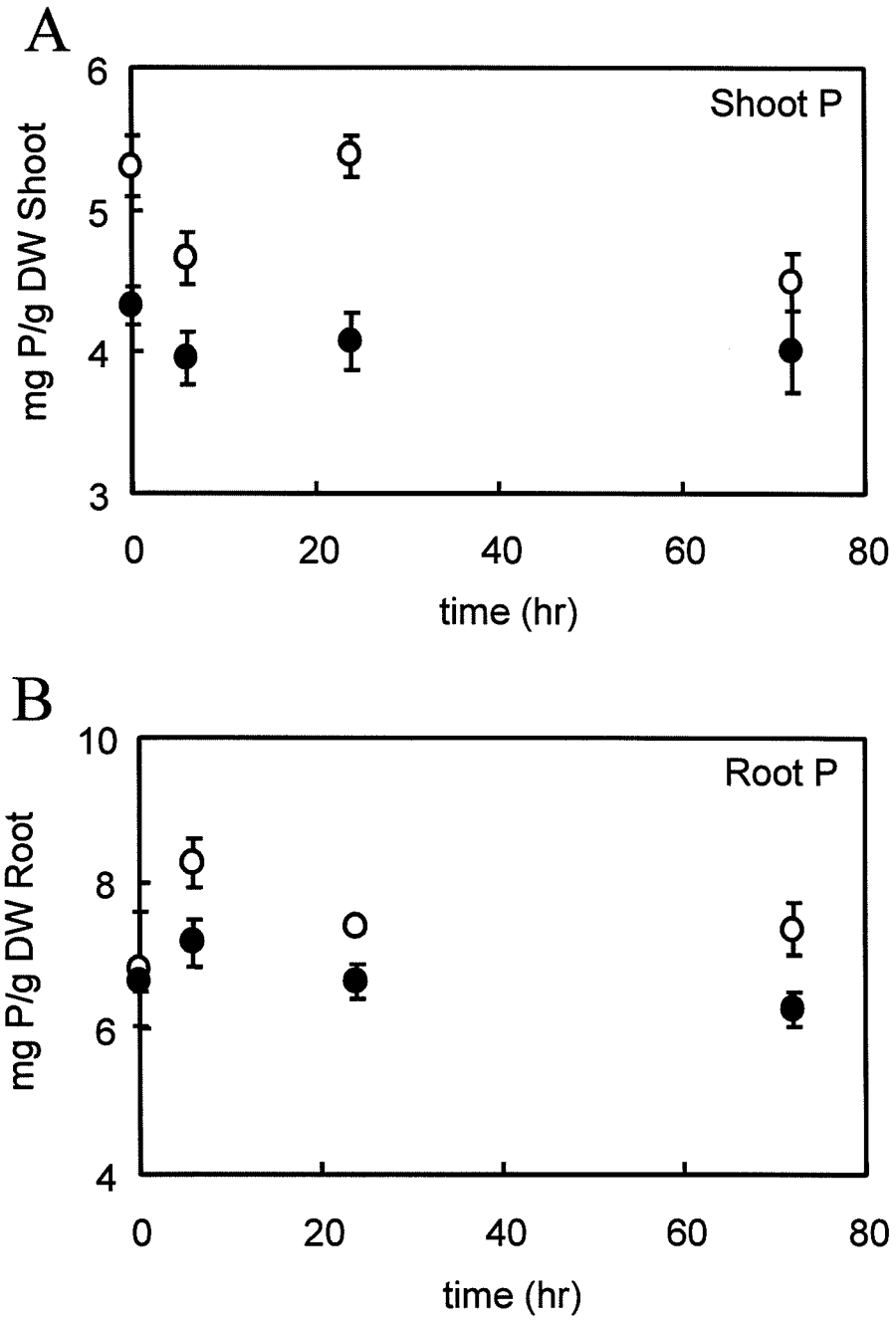

Figure 3-20. Kinetics of the changes in P concentration in the shoots (A) and roots (B) of Arabidopsis (open circles) and Thellungiella (closed circles) plants during a 72 h treatment with 100 mM NaCl in hydroponic growth solution. Ion concentrations of single plants were determined by ICP-OES. Values are the mean ± SE (n=9).

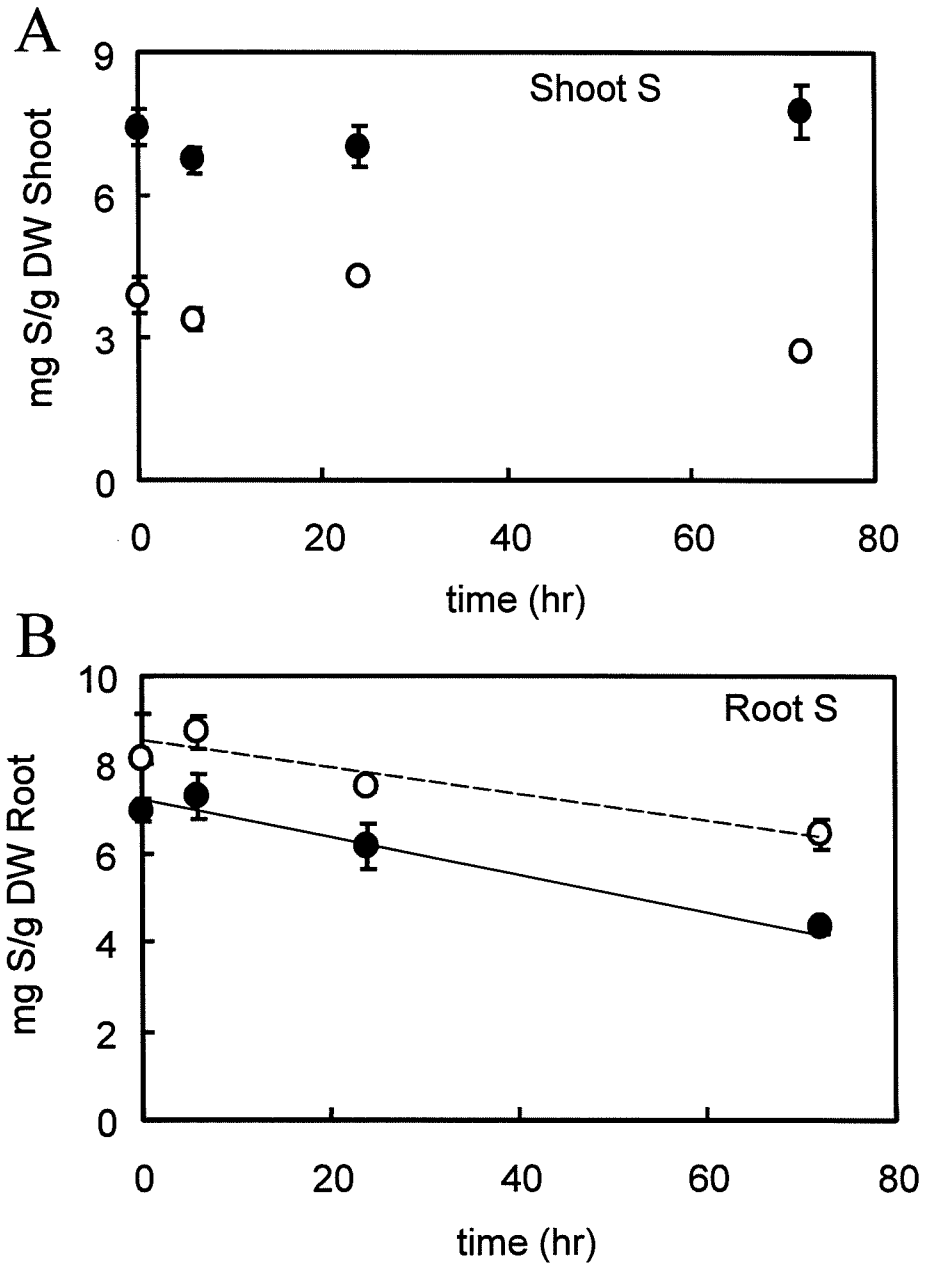

Figure 3-21. Kinetics of the changes in S concentration in the shoots (A) and roots (B) of Arabidopsis (open circles) and Thellungiella (closed circles) plants during a 72 h treatment with 100 mM NaCl in hydroponic growth solution. Ion concentrations of single plants were determined by ICP-OES. Values are the mean ± SE (n=9).

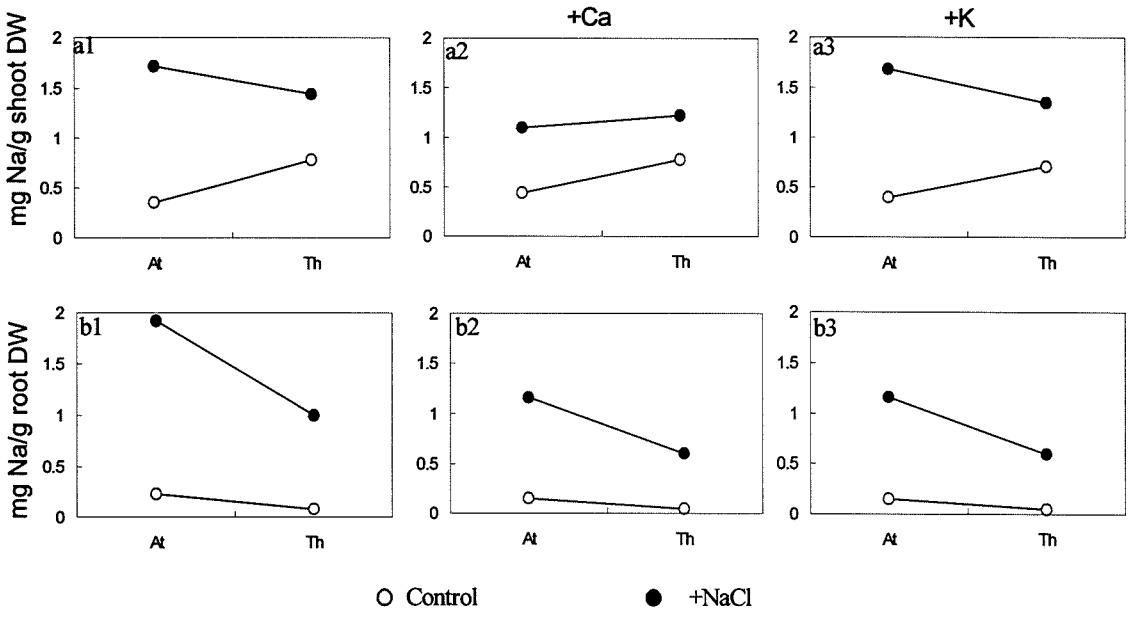

Figure 3-22. Effects of external Ca (+Ca) and K (+K) on Na concentrations in Arabidopsis and Thellungiella before and after 25 h treatment with 100 mM NaCl. Mean values of absolute ion concentrations (mg/g DW) in Arabidopsis and Thellungiella with (+NaCl: closed circles) and without (control: open circles) salt treatment were plotted against species. The data with additional 5 mM Ca^{2+} or 10 mM K^{+} were plotted in separate graphs. Na concentrations in the roots and the shoots were plotted separately. a1: shoot Na without supplement; a2: shoot Na with external Ca; a3: shoot Na with external K; b1: root Na without supplement; b2: root Na with external Ca; b3: root Na with external K.

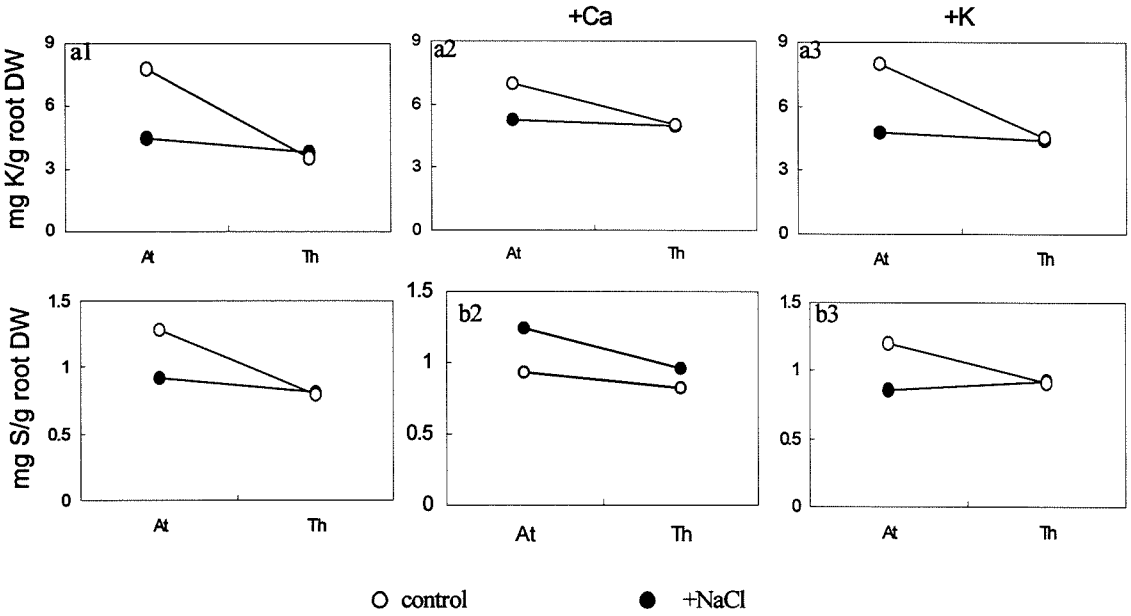

Figure 3-23. Effects of external Ca (+Ca) and K (+K) on salt-induced K and S concentrations in the roots of Arabidopsis and Thellungiella. Mean values of absolute ion concentrations (mg/g DW) in Arabidopsis and Thellungiella with (+NaCl: closed circles) and without (control: open circles) salt treatment were plotted against species. The data with additional 5 mM Ca^{2+} or 10 mM K^{+} were plotted in separate graphs. Na concentrations in the roots and the shoots were plotted separately. a1: root K without supplement; a2: root K with external Ca; a3: root K with external K; b1: root S without supplement; b2: root S with external Ca; b3: root S with external K.

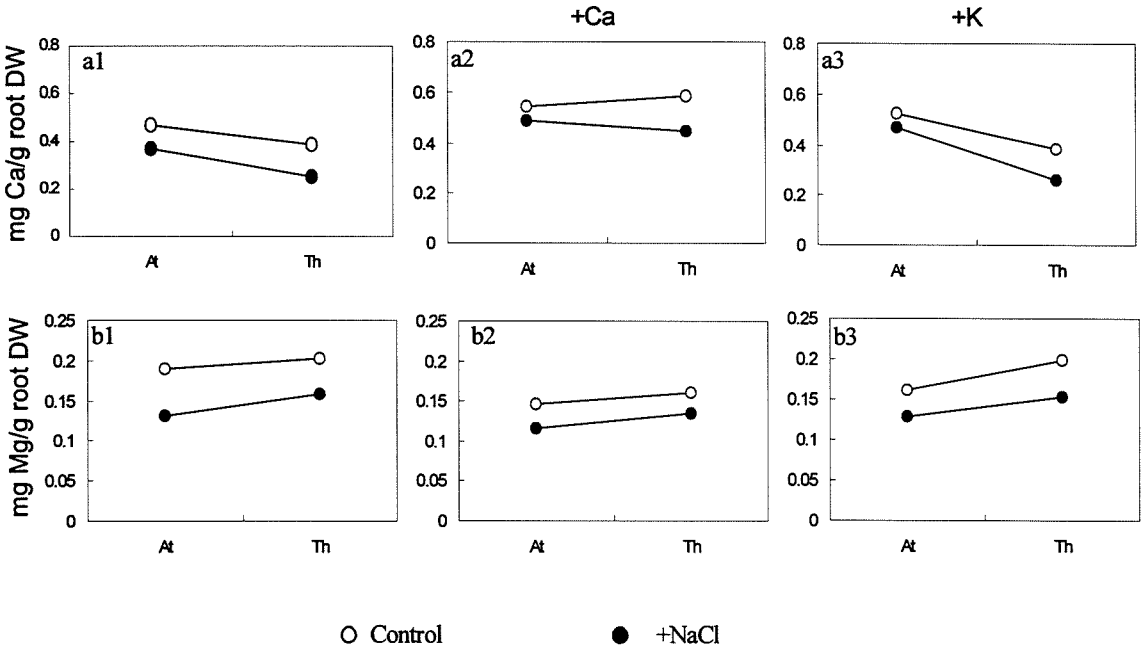

Figure 3-24. Effects of external Ca (+Ca) and K (+K) on root Ca and Mg concentrations in Arabidopsis and Thellungiella. Mean values of absolute ion concentrations (mg/g DW) in Arabidopsis and Thellungiella with (+NaCl: closed circles) and without (control: open circles) salt treatment were plotted against species. The data with additional 5 mM Ca^{2+} or 10 mM K^{+} were plotted in separate graphs. Na concentrations in the roots and the shoots were plotted separately. a1: root Ca without supplement; a2: root Ca with external Ca; a3: root Ca with external K; b1: root Mg without supplement; b2: root Mg with external Ca; b3: root Mg with external K.

Table 3-1. Treatments applied for the determination of ion profiles

Treatment name	Solution components
'Control'	MNS (see Table 2-1 and Table 2-2 in Section 2.2.1)
'+NaCl'	MNS + 100 mM NaCl
'+KCl'	MNS + 10 mM KCl
'+$CaCl_2$'	MNS + 5 mM $CaCl_2$
'+NaCl +KCl'	MNS + 100 mM NaCl + 10 mM KCl
'+NaCl +$CaCl_2$'	MNS + 100 mM NaCl + 5 mM $CaCl_2$

Table 3-2. Direction and significance of differences in net K accumulations between treatments or species. Symbols in brackets indicate whether the ratio was larger (>) or smaller (<) than 1 or not significant (=). Numbers are p values obtained in t tests, where pairs represented plants batches grown and treated in parallel, and replicates represented consecutively grown and treated plant batches. Significant p values are in bold. For further explanation see footnotes.

	Shoot				Root			
	Comparison between treatments (Salt / Control)		Comparison between species (Thellungiella / Arabidopsis)		Comparison between treatments (Salt / Control)		Comparison between species (Thellungiella / Arabidopsis)	
	A.thal.	T.halo.	'Control'	'+Na'	A.thal.	T.halo.	'Control'	'+Na'
Short term								
Absolute values	**0.0503 <** [(1)]	0.9572 =	0.5047 = [(2)]	0.1190 =	**0.0510 <**	0.6829 =	**0.0205 <**	0.8323 =
Relative changes	**0.0387 <**	0.9020 =	Set to =	0.2945 =	**0.0153 <**	0.5807 =	Set to =	**0.0498 >**
Long term								
Absolute values	0.2379 =	0.2051 =	0.6509 =	0.8212 =	0.4325 =	0.2339 =	0.6039 =	0.6747 =
Relative changes	0.3067 =	0.3672 =	Set to =	0.8430 =	0.1636 =	0.2536 =	Set to =	0.3351 =

(1) Read as: Salt treated Arabidopsis plants had a signifcantly lower absolute K concentration than control Arabidopsis plants with a p value of 0.0503.

(2) Read as: In control conditions Thellungiella had a similar absolute K concentration as Arabidopsis.

Table 3-3. Direction and significance of differences in net Ca accumulations between treatments or species. Symbols in brackets indicate whether the ratio was larger (>) or smaller (<) than 1 or not significant (=). Numbers are p values obtained in t tests, where pairs represented plant batches grown and treated in parallel, and replicates represented consecutively grown and treated plant batches. Significant p values are in bold.

	Shoot				Root			
	Comparison between treatments (Salt / Control)		Comparison between species (Thellungiella / Arabidopsis)		Comparison between treatments (Salt / Control)		Comparison between species (Thellungiella / Arabidopsis)	
	A.thal.	T.halo.	'Control'	'+Na'	A.thal.	T.halo.	'Control'	'+Na'
Short term								
Absolute values	0.1790 =	0.6267 =	**0.0003 <**	**0.0116 <**	**0.0100 <**	**0.0028 <**	0.3958 =	0.2753 =
Relative changes	0.1838 =	0.6826 =	Set to =	0.7494 =	**0.0210 <**	**0.0008 <**	Set to =	0.1860 =
Long term								
Absolute values	0.3688 =	**0.0087 <**	**0.0282 <**	**0.0315 <**	**0.0500 <**	**0.0075 <**	0.2782 =	**0.0545 <**
Relative changes	0.4304 =	**0.0013 <**	Set to =	0.1068 =	**0.0130 <**	**0.0029 <**	Set to =	**0.0105 <**

Table 3-4. Direction and significance of differences in net Mg accumulations between treatments or species. Symbols in brackets indicate whether the ratio was larger (>) or smaller (<) than 1 or not significant (=). Numbers are p values obtained in t tests, where pairs represented plants batches grown and treated in parallel, and replicates represented consecutively grown and treated plant batches. Significant p values are in bold.

	Shoot				Root			
	Comparison between treatments (Salt / Control)		Comparison between species (Thellungiella / Arabidopsis)		Comparison between treatments (Salt / Control)		Comparison between species (Thellungiella / Arabidopsis)	
	A.thal.	T.halo.	'Control'	'+Na'	A.thal.	T.halo.	'Control'	'+Na'
Short term								
Absolute values	0.1153 =	0.8805 =	**0.0004 <**	**0.0183 <**	**0.0241 <**	0.0615 =	0.2167 =	**0.0110 >**
Relative changes	0.1226 =	0.9080 =	Set to =	0.5820 =	**0.0136 <**	**0.0367 <**	Set to =	**0.0152 >**
Long term								
Absolute values	0.3081 =	0.5144 =	**0.0024 >**	0.6132 =	**0.0039 <**	**0.0076 <**	0.1084 =	0.8522 =
Relative changes	0.1876 =	0.4538 =	Set to =	0.4286 =	**0.0000 <**	**0.0100 <**	Set to =	0.4111 =

Table 3-5. Direction and significance of differences in net P accumulations between treatments or species. Symbols in brackets indicate whether the ratio was larger (>) or smaller (<) than 1 or not significant (=). Numbers are p values obtained in t tests, where pairs represented plant batches grown and treated in parallel, and replicates represented consecutively grown and treated plant batches. Significant p values are in bold.

	Shoot				Root			
	Comparison between treatments (Control / Salt)		Comparison between species (Thellungiella / Arabidopsis)		Comparison between treatments (Control / Salt)		Comparison between species (Thellungiella / Arabidopsis)	
	A.thal.	T.halo.	'Control'	'+Na'	A.thal.	T.halo.	'Control'	'+Na'
Short term								
Absolute values	**0.0220 <**	0.7282 =	**0.0098 <**	0.3560 =	**0.0002 <**	0.4683 =	**0.0027 <**	0.1770 =
Relative changes	**0.0231 <**	0.7096 =	Set to =	0.3270 =	**0.0000 <**	0.5254 =	Set to =	0.2659 =
Long term								
Absolute values	0.2173 =	0.9934 =	0.1501 =	0.1139 =	0.3764 =	0.3899 =	0.7454 =	0.2079 =
Relative changes	0.2162 =	0.9238 =	Set to =	0.2971 =	0.6287 =	0.3848 =	Set to =	0.9406 =

Table 3-6. Direction and significance of differences in net S accumulations between treatments or species. Symbols in brackets indicate whether the ratio was larger (>) or smaller (<) than 1 or not significant (=). Numbers are p values obtained in t tests, where pairs represented plant batches grown and treated in parallel, and replicates represented consecutively grown and treated plant batches. Significant p values are in bold.

	Shoot				Root			
	Comparison between treatments (Salt / Control)		Comparison between species (Thellungiella / Arabidopsis)		Comparison between treatments (Salt / Control)		Comparison between species (Thellungiella / Arabidopsis)	
	A.thal.	T.halo.	'Control'	'+Na'	A.thal.	T.halo.	'Control'	'+Na'
Short term								
Absolute values	0.1243 =	0.8543 =	0.2222 =	0.1791 =	**0.0272 <**	0.6672 =	**0.0384 <**	0.3477 =
Relative changes	0.1011 =	0.7973 =	Set to =	0.4813 =	**0.0124 <**	0.6204 =	Set to =	0.0688 =
Long term								
Absolute values	0.2102 =	0.8109 =	0.0804 =	0.1486 =	0.7195 =	0.7310 =	0.5605 =	0.3001 =
Relative changes	0.2645 =	0.5304 =	Set to =	0.6813 =	0.7566 =	0.7942 =	Set to =	0.7118 =

Table 3-7. Direction and significance of differences in shoot / root ratios of ions between treatments or species. Symbols in brackets indicate whether the ratio was larger (>) or smaller (<) than or equal (=) to 1. Numbers are p values obtained in t tests, where pairs represented plants batches grown and treated in parallel, and replicates represented consecutively grown and treated plant batches.

	Comparison between treatments (Salt / Control)		Comparison between species (Thellungiella / Arabidopsis)	
	A.thal.	T.halo.	'Control'	'+Na'
K				
Short term	0.1039 =	0.6956 =	**0.0432** >	0.1658 =
Long term	0.7856 =	0.5617 =	0.9785 =	0.8282 =
Ca				
Short term	**0.0465** >	0.0794 =	**0.0094** <	**0.0386** <
Long term	0.9167 =	**0.0231** <	**0.0089** <	0.0624 =
Mg				
Short term	0.1511 =	0.1018 =	**0.0001** <	**0.0145** <
Long term	0.5070 =	0.4349 =	**0.0248** <	0.5322 =
P				
Short term	0.4264 =	0.5640 =	0.0698 =	0.8258 =
Long term	0.0594 =	0.7029 =	0.3177 =	0.1289 =
S				
Short term	0.1725 =	0.8680 =	**0.0108** >	0.1930 =
Long term	0.1324 =	0.6279 =	0.2948 =	0.6655 =

Table 3-8. Kinetic analysis of changes in net ion accumulations: fitted equations and parameters.

Parameters extracted	Fitted equation	R^2	Fig.
Net K loss from the roots	*At*: $f(t) = 50.7 - 0.33t$	0.94	5B
(mg K/ g root DW/min)	*Th*: $f(t) = 44.1 - 0.10\,t$	0.90	
Net Mg loss from the	*At*: $f(t) = 0.27e^{-0.25t}+1.00e^{-0.0010t}$	1	13A
roots (h)	*Th*: $f(t) = 0.63e^{-0.1487t}+1.47e^{-0.0015t}$	1	
Net S loss from the roots	*At*: $f(t) = 8.43 - 0.028t$	0.87	21B
(mg S/g root DW/min)	*Th*: $f(t) = 7.21 - 0.040t$	0.97	

Table 3-9. ANOVA output showing significances of differences in ion concentrations between Arabidopsis and Thellungiella, ion concentrations before and after treatment with salt, and ion concentrations with and without Ca or K supplement, and the inter-dependence of the three factors. 3-way ANOVA was applied to ion concentrations obtained from 3 independent replicates. 'df' stands for degree of freedom. F stands for ratio of MS and Residual Error. P values in the 'Sig.' column indicate the significance of the differences. This table is divided into 3 subtables, A: K and Na; B: Ca and Mg; C: P and S.

Subtable A		Shoot K		Root K		Shoot Na		Root Na	
	df	F	Sig.	F	Sig.	F	Sig.	F	Sig.
Species	1	7.104	0.014	15.400	0.001	0.527	0.475	58.816	0.000
Salt	1	2.540	0.124	8.289	0.008	42.164	0.000	498.00	0.000
Supplement	2	0.695	0.509	0.759	0.479	0.785	0.468	15.162	0.000
Species * Salt	1	1.391	0.250	8.564	0.007	3.941	0.059	28.113	0.000
Species * Supplement	2	0.110	0.896	0.667	0.522	0.296	0.746	3.386	0.051
Salt * Pretreat	2	0.356	0.704	0.258	0.775	1.249	0.305	10.610	0.000
Species * Salt * Supplement	2	0.101	0.904	0.372	0.693	0.376	0.691	2.124	0.141
Subtable B		Shoot Ca		Root Ca		Shoot Mg		Root Mg	
	df	F	Sig.	F	Sig.	F	Sig.	F	Sig.
Species	1	116.90	0.000	7.247	0.013	108.90	0.000	5.596	0.026
Salt	1	1.275	0.270	8.681	0.007	0.313	0.581	17.419	0.000
Supplement	2	0.140	0.870	6.509	0.006	0.245	0.784	3.778	0.037
Species * Salt	1	0.394	0.536	0.874	0.359	0.098	0.757	0.010	0.921
Species * Supplement	2	0.269	0.766	2.262	0.126	0.149	0.863	0.255	0.777
Salt * Pretreat	2	0.192	0.826	0.056	0.946	0.019	0.981	0.525	0.598
Species * Salt * Supplement	2	0.030	0.971	0.039	0.962	0.196	0.824	0.176	0.839
Subtable C		Shoot P		Root P		Shoot S		Root S	
	df	F	Sig.	F	Sig.	F	Sig.	F	Sig.
Species	1	20.311	0.000	10.199	0.004	27.751	0.000	35.787	0.000
Salt	1	0.837	0.369	3.287	0.082	0.003	0.960	29.601	0.000
Supplement	2	0.590	0.562	0.493	0.617	0.244	0.786	0.357	0.703
Species * Salt	1	0.529	0.474	0.162	0.691	0.602	0.446	20.593	0.000
Species * Supplement	2	0.927	0.409	0.084	0.920	1.041	0.369	2.702	0.087
Salt * Pretreat	2	0.434	0.653	0.391	0.680	0.163	0.851	0.281	0.757
Species * Salt * Supplement	2	0.250	0.781	0.168	0.846	0.228	0.798	0.952	0.400

Chapter 4 Transcriptional profiling

This chapter presents the results from microarray experiments using an Arabidopsis membrane transporter microarray (AMT array, Maathuis et al., 2003) to compare the transcriptional profiles of membrane transporters between Arabidopsis and Thellungiella. The results are compared with the results from ion measurement to understand the involvement of transporters in ion homeostasis during salt stress.

4.1 Introduction

DNA microarray technology is one of the most efficient methods to achieve genome-wide gene expression profiling. It substantially improved the sensitivity and throughput of transcriptional expression screening (van Hal et al., 2000).

In the Introduction section, I will first describe the principle of microarray technology and its advantages. Then I will briefly introduce the methods used for analyzing the array results. Next I will give a brief summary of the current knowledge on genes that are transcriptionally regulated by salt stress in Arabidopsis and Thellungiella plants. Finally I will explain the experimental design and the questions addressed in this study.

4.1.1 DNA microarray

DNA microarray technology was first developed by Patrick Brown and his colleagues at Stanford University in 1995 (Shena et al., 1995). In 1997 they applied DNA microarray technique to genome wide parallel genetic and gene expression analysis in Yeast (Lashkari et al., 1997). The technique is based on hybridization of fluorescently labelled cDNA to a high-density array of immobilized probe sequences, each corresponding to a specific gene. The DNA probes (primarily full-length cDNA or synthesized oligonucleotides) are attached to a solid support, usually a glass slide. Fluorescently labelled RNA or cDNA samples are prepared from tissue samples and then hybridized

to the complementary DNA probes on the array. The array is then scanned to measure fluorescence intensity at each probe location (spot). The signal intensities are converted to a quantitative read-out of gene expression levels, which can be compared between different samples and further analysed to correlate the expression patterns and their variation with cellular development, physiology and function (Harrington et al., 2000).

There are two basic types of microarrays — cDNA microarrays and high density oligonucleotide microarrays. They differ in the source of the probe sequences. For cDNA microarray, full length or partial cDNA sequences are cloned and PCR amplified from cDNA libraries. For oligonucleotide arrays smaller fragments (25-60 bp) are synthesized *de novo* using existing sequence information. Probes can either be spotted onto the arrays and covalently linked to the surface or directly 'grown' on the slide using photolithographic techniques.

The RIKEN Arabidopsis full-length (RAFL) cDNA microarray is a typical example of a cDNA microarray, and has been used in studying expression profiles of Arabidopsis genes under varous stress conditions, such as drought, cold and salt stress (Seki et al., 2001; Seki et al., 2002), high light stress (Kimura et al., 2003), UV– C (Narusaka et al., 2003), or rehydration treatment after dehydration (Oono et al., 2003), as well as various treatments including abscisic acid (ABA) (Rabbani et al., 2003), ethylene (Narusaka et al., 2003), jasmonic acid (JA) (Narusaka et al., 2003), salicylic acid (SA) (Narusaka et al., 2003), reactive oxygen species (ROS)-inducing compounds such as paraquat and rose bengal (Narusaka et al., 2003), proline (Pro) (Satoh et al., 2002), and inoculation with pathogens (Narusaka et al., 2003).

Oligonucleotide microarrays are commercially available. For example, Affymetrix, Inc. (Santa Clara, USA) provides the whole genome Arabidopsis GeneChip® ATH1 array representing 24, 000 genes. Agilent Technologies, Inc. (Palo Alto, USA) supplies

oligonucleotide probes and hybridisation kits similar to the Affymetrix ATH1 array. Qiagen Ltd. (West Sussex, UK) provides Arabidopsis whole genome or customized oligonucleotide probes for array spotting. Prof. David Galbraith at the Department of Plant Sciences, University of Arizona, USA, has spotted whole genome Arabidopsis arrays using the Qiagen-Operon Arabidopsis Genome Array Ready Oligo Set (AROS). This array is available for academic use at low price. The data presented in this chapter was obtained from an oligonucleotide array, the Arabidopsis Membrane Transporter (AMT) array (Maathuis et al., 2003, see below).

The major advantages of DNA microarray technology compared to conventional techniques such as Northern blotting and dot blots, are the small size of the array, a high sensitivity due to the usage of fluorescent dyes, the possibility for parallel screening of larger numbers of genes and the opportunity to use small amounts of starting material. The scale of gene expression analysis is extended not only by the simultaneous analysis of large numbers of genes, but also because microarrays can be produced in series facilitating comparative analysis of a large number of samples (van Hal et al., 2000).

4.1.2 The Arabidopsis Membrane Transporter Array (AMT array)

Arabidopsis Membrane Transporter array (AMT array) was designed by an international consortium led by Dr. Anna Amtmann to study Arabidopsis membrane transporter expression (Maathuis et al., 2003). It is an oligonucleotide microarray with 1250 probes for 1153 genes including 57 control genes and 1096 genes encoding all annotated Arabidopsis transporter genes and all non-annotated genes with six or more transmembrane spanning domains (Figure 4-1).

The probe sequences were synthesized and spotted onto glass slides by MWG Biotech AG (Ebersberg, Germany). All probes are spotted in duplicate on each array. Gene-specific 50 mer probes were designed according to a specific region in the 3' end of

each gene. The use of 50 mer probes rather than longer cDNAs allowed the design of gene-specific probes for nearly all genes represented on the array except for eight pairs of genes, whose sequences were too similar to ensure specific hybridization signals: At1g07810 and At1g07670 (ECA1/4 95.6% homology), At3g28710 and At3g28715 (VHA-d1/d2, 99.9% homology), At2g16130 and At2g16120 (STP 31/32, 92.7% homology), At5g43350 and At5g43370 (PHT1.1/1.2, 97.7% homology), At1g26730 and At1g35350 (putative proteins in DASS family, 88.6% homology), At3g03700 and At3g04440 (hypothetical proteins, 99.8% homology), At1g18010 and At1g18000 (hypothetical proteins, 100%), At4g37680 and At4g38320 (alternative splice forms of a putative protein) (Maathuis et al., 2003). Most genes are represented on the AMT array by one probe. For 54 ABC transporters, two or three oligonucleotide probes were designed (Maathuis et al., 2003).

The AMT array has several advantages for this study. Ion homeostasis and ion transporter expression control are the main interests of this study. Therefore a membrane transporter array fulfils the needs for monitoring expression profiles of membrane transporters. It does not produce data on other genes with stronger transcriptional regulation by salt such as transcription factors, kinases and phosphatases, that would unnecessarily increase the complexity of the data. The 50 mer probes are long enough to allow cross-species hybridization with Thellungiella cDNA but are still specific enough to distinguish between different members in the same gene family.

Sample mRNA was labelled with fluorescent Cyanine dyes during oligo-dT primed reverse transcription. Samples with different treatments were labelled with differently coloured dyes. Equal amounts of differentially labelled cDNA samples were combined and hybridized to the array. The expression difference of each gene was determined by the difference in the signal intensity produced by the two dyes in each spot.

4.1.3 Novel Methods for Array Analysis— RP and iGA

The best methods for array scanning, extraction, normalization, and data analysis have not been determined (Finkelstein et al., 2002). There are numerous methods and computer software available to date for microarray imaging, data extraction, normalization and data mining, e.g. GeneSpring (Agilent Inc., Palo Alto, USA), DNA Chip Analyzer (dChip) (Li and Wong, 2001), and TIGR Multiple Experiment Viewer (MEV) (Saeed et al., 2003). Novel microarray data analysis methods, Rank Product (RP) and iterative Group Analysis (iGA) were developed in Glasgow (Breitling et al., 2004a; Breitling et al., 2004b). The array results in this chapter were analyzed and extracted using these methods.

4.1.3.1 Rank Product

Rank Product (RP) is a novel method for identifying differentially expressed genes that is based on calculating rank products from replicate experiments. For each replicate experiment genes are ranked according to their fold changes. Ranks are then multiplied over all replicates and the entire gene list is sorted according to the RP. A significance is assigned to each rank product based on the likelihood to obtain a certain RP value from a large number of random permutations. In this way not only the significance of the expression change of the genes, but also the consistency of such changes are taken into account when determining the importance of gene expression changes. The method is fast and simple. At the same time, it provides a straightforward and statistically stringent way to determine the significance of the fold change for each gene and allows control of the false-detection rate and family-wise error rate in the multiple testing situation of a microarray experiment (Breitling et al., 2004b).

4.1.3.2 Iterative Group Analysis

Iterative Group Analysis (iGA, (Breitling et al., 2004a)) is based on a comprehensive hypergeometric statistics detecting concerted changes in functional categories of genes. The functional categories can be derived from various sources (e.g. GeneOntology assignments http://www.geneontology.org, BLAST result key words, literature extracts) and the detection algorithm will automatically determine the genes in each category that are as a group, most likely to be differentially expressed. The smallest p value that is obtained by a group of genes within a functional category is assigned to the category. This, however, does not mean that other members of the functional category are not differentially expressed. It simply identifies the sub-group of genes in the category which has a position in the RP ranked list that is least likely to occur randomly. The iGA approach not only provides useful information on the physiological function of gene expression changes but also can enhance the sensitivity of the detection of differentially expressed genes, especially for small, noisy data sets (Breitling et al., 2004a).

4.1.4 Current knowledge on salt-affected gene expression

Gene expression has been studied in plants subjected to salt stress as well as other abiotic stresses such as drought, cold, high-light and oxidative stress, nutritional stress (K, P, S deficiency) and treatments with phytohormones i.e. abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA) (Armengaud et al., 2004; Buchner et al., 2004; Hawkesford, 2000; Kreps et al., 2002; Liu, 2005; Maathuis et al., 2003; Narusaka et al., 2004; Popova et al., 2003; Rabbani et al., 2003; Seki et al., 2003; Seki et al., 2001; Seki et al., 2002; Seki et al., 2004; Taji et al., 2004a).

According to the studies by Shinozaki and colleagues with the RAFL cDNA microarray covering 7000 genes (Seki et al., 2002) the stress regulated genes in glycophytic plants

such as Arabidopsis can be classified into two general groups. The first group includes functional proteins such as membrane transporters, osmoticum-related proteins, detoxification enzymes, KIN proteins, late-embryogenesis-abundant (LEA) proteins, heat shock proteins, carbohydrate-metabolism-related proteins, senescence-related proteins, proteases and inhibitors, and lipid transfer proteins. The second group contains regulatory proteins, such as protein factors, transcription factors, protein kinases and phosphatases, and other signaling molecules.

In a study of transcriptomic changes of cultured Arabidopsis T87 cells using the RAFL arrays, Seki et al. (2004) found that after 5 h treatment with 100 mM NaCl 17 genes were induced and 41 genes were suppressed. Analysis of overlap among the genes induced by mannitol, NaCl and ABA revealed that 11 genes were induced by all three stresses. Five of them were well-known stress-inducible genes, *COR78*, *KIN1*, *KIN2*, *COR47* and *ERD10* (Bohnert et al., 1995; Bray, 1997; Ingram and Bartels, 1996; Kiyosue et al., 1994). The other genes were also mostly defence-related. Among them 10 genes contained ABRE as well as DRE-core in their promoter region, suggesting that the mannitol- and salinity-responsible gene regulation could be regulated via a common signal transduction pathway possibly involving ABA. Overlap between salt and osmotic stress regulated expression changes was also found in Thellungiella by Wong et al. (2006) with a Thellungiella cDNA microarray. However many genes regulated by both drought and salt in Arabidopsis were not affected by salt in Thellungiella (Wong et al., 2006). Genes involved in ABA synthesis and ABA responsiveness were shown to be expressed more abundantly in Thellungiella than in Arabidopsis (Gong et al., 2005), suggesting that it might not be necessary for Thellungiella to regulate these genes during salt stress.

In a study with the first Arabidopsis Affymetrix GeneChip® covering 8,000 genes, Kreps et al. (2002) discovered 22 genes that were exclusively regulated in roots of Arabidopsis by salt (27 h treatment with 100 mM NaCl), within which the largest category (50%) were related to oxidative stress enzymes i.e. glutathione reductase and cytochrome P450. The top three up-regulated genes in response to salt stress in both leaves and roots of Arabidopsis were *COR78*, an unknown protein and a LEA protein. Kreps et al. (2002) also found differential regulation of several transporter genes. In the roots of Arabidopsis plants, a putative Na/H exchanger, *CHX17* was up-regulated, and an auxin transporter *EIR1*, a high-affinity nitrate transporter *NRT2* and a Ca pump *AtACA4* were down-regulated after 3 h salt treatment. More transporter genes were induced in the leaves by salt including two nitrate transporters, *PIP2B*, an ammonium transporter *ATM1,2*, a K channel *AKT2/3*, two sugar transporters *ERD6* and *SUC2* and a sulphate transporter *ATST1*.

Maathuis et al. (2003) analysed transcriptional changes of root transporters in Arabidopsis induced by cation stresses, including salt stress, using the AMT array. Relatively high numbers of CNGC, glutamate receptor and anion transporter encoding genes were specifically affected by treatment with 80 mM NaCl. It has been postulated that CNGCs and glutamate receptors contribute to Na uptake in their capacity of non-selective cation channels (Demidchik et al., 2002; Talke et al., 2003). The primary H^+ pump *AHA2* was consistently modulated at the transcriptional level, and is likely to play a crucial role in energizing Na extrusion at the root-soil boundary by providing the necessary PMF to drive H-coupled Na export. Several Ca pumps showed up-regulation at various time points during 96 h salt treatment. However expression levels of *ACA4* which was shown to decrease after salt treatment by Kreps et al. (2002), did not change in this study. Most of the subunits of vacuolar ATPases were up-regulated in response

to salt, particularly in the later stages (24 – 96 h) of the treatment. Transcripts of *NHX1* and *NHX3* showed transient increase during salt stress. However the expression of the plasma membrane Na/H antiporter *SOS1* (*NHX7*) was not affected by salt. Several Ca/H antiporters showed transient weak up-regulation by salt treatment, but *CAX3*, *CAX5* and *CAX6* were strongly down-regulated by salt. Several CHX isoforms were significantly and consistently down-regulated, especially *CHX10* and *CHX15*. Salt stress induced an initial down-regulation, which was followed by a substantial upregulation of many aquaporin isoforms. These expression changes occurred earlier for the plasma membrane-expressing isoforms (PIP subfamily) than for the tonoplast-expressing TIP subfamily. Within the ABC transporter family (a large group that contains 129 members), *MDR13*, *PUP3* and a cluster of ATH subfamily (*ATH13-16*) were down-regulated during salt treatment, whereas *PDR7* and *PDR8* transcripts were up-regulated by salt stress. Salt stress also affected nitrate nutrition judging by the considerable down-regulation of NAR2-like and *NRT2,1* transcripts.

Several studies have compared gene expression between Arabidopsis and Thellungiella (Taji et al., 2004; Gong et al., 2005; Wong et al., 2006). In a study using the RAFL arrays, Taji et al. (2004) reported that after 2 h treatment with 250 mM NaCl only 6 genes were induced by salt stress in Thellungiella, whereas 40 genes were induced in Arabidopsis plants. Several other groups (Gong et al., 2005; Wong et al., 2006) also found that fewer genes were regulated by salt in Thellungiella than in Arabidopsis. The six salt-induced genes in Thellungiella plants encode myoinositol-1-phosphate synthases, a galactinol synthase, a desiccation-induced protein, RD20, a LEA-like protein and a protein kinase. Taji and colleagues also directly compared gene expression in Thellungiella and Arabidopsis plants grown in low-salt conditions by hybridizing the full-length Arabidopsis cDNA microarrays with mRNA from both species. A number of

abiotic or biotic stress-inducible genes were constitutively more strongly expressed in Thellungiella, for example SOD, NCED2, chitinase, plant defensin1.2 (PDF1.2), P5CS, SOS1, P-protein associated with nitric oxide (NO) production and β-glucosidase. Gong et al. (2005) identified a group of genes that were down-regulated in Arabidopsis but either unchanged or up-regulated in Thellungiella after salt stress, which indicates that in contrast to Arabidopsis Thellungiella is able to resume growth and/or initiate damage repair quickly after salt stress. In another study, after a total of 21 days treatment with up to 300 mM NaCl, Wong et al. (2006) found that only 22 genes out of 3628 genes represented on the Thellungiella cDNA array had changed their expression in shoots of Thellungiella. Generally, these studies identified few salt regulated transcripts encoding membrane transporters.

However, there is evidence that salt-tolerant species such as Thellungiella are able to restrict accumulation of Na in photosynthetic tissues (Inan et al., 2004; Taji et al., 2004b; Volkov et al., 2004). In Chapter 2 of this thesis, lower unidirectional Na influx into the root cells of Thellungiella was demonstrated. Therefore ion transporters or genes controlling ion transporters are likely to be direct contributors to salt tolerance in Thellungiella, whereas genes involved in anti-oxidative-stress and detoxification may play a supportive role in salt stress or general stress adaptation.

4.1.5 Experimental design and questions addressed

Analysis of ion transport in Arabidopsis and Thellungiella plants revealed that after salt treatment both uptake of Na and loss of K were slower in Thellungiella plants than in Arabidopsis (Chapter 2). Lower Na uptake in Thellungiella is due to lower unidirectional Na influx into the roots of Thellungiella (Chapter 2). Possible reasons for the above differences in ion transport between Arabidopsis and Thellungiella are that (i) the respective ion transporters are expressed at different levels in Arabidopsis and

Thellungiella, (ii) the expression of ion transporter genes are differently regulated by salt stress in the two species, (iii) the post-translational regulation of ion transporters differs between the two species, or (iv) the protein structure of the ion transporters is different in Arabidopsis and Thellungiella which affects their ion transport capacities. To address some of these issues transcriptional profiles of ion transporters in Arabidopsis and Thellungiella plants with and without salt treatment were analyzed using the AMT array.

In contrast to the previous studies RNA was isolated separately from roots and shoots of Arabidopsis and Thellungiella plants. RNA samples from the same species with and without salt treatment (24 h treatment with 100 mM NaCl in MNS) were hybridized together. Therefore 4 types of array were produced (Table 4-1). Array hybridization with shoot samples was repeated 3 times, and array hybridization with root samples was repeated 4 times, always using RNA samples from independent batches of plants. After normalization the obtained intensity profiles (where the intensity is the measure of the fluorescent signal from one dye in a particular spot) are suitable for comparison not only between simultaneously hybridized sample pairs but also between samples that are hybridized to different arrays. Three types of comparisons were made with the normalized intensity profiles (Table 4-1):

1. Intensity distribution curve. The numbers of genes displaying an intensity above certain values were plotted against these values for each sample. In this way overall expression levels of transporter genes were compared between Arabidopsis and Thelluniella. These curves could be used to assess the gene similarity between Arabidopsis and Thellungiella. The general transcriptional response of membrane transporter genes to salt stress in each species was also displayed by these curves.

2. Difference of transporter expression between the two species. The normalized intensity profiles of RNA samples from the same tissue (root or shoot) with the same treatment ('control' or '+NaCl') were compared between Arabidopsis and Thellungiella. The individual transcripts or functional groups of transcripts that were more abundant in Thellungiella than in Arabidopsis grown in control condition or after salt treatment were identified with RP and iGA. Transcripts with lower abundance in Thellungiella than in Arabidopsis were identified too. However it is unclear whether the lower signal of the transcripts from Thellungiella compared to Arabidopsis is due to lower expression or to low cDNA homology (note that the array probes were designed for Arabidopsis).
3. Salt-induced expression changes in each species. The fold changes of transcripts in response to salt stress were calculated to identify the genes induced or depressed by salt stress. The transporter genes commonly regulated by salt in Arabidopsis and Thellungiella were identified, together with potentially more interesting genes that were specifically regulated by salt in Thellungiella.

4.2 Materials and Methods

4.2.1 Plant growth and treatment for AMT array experiment

Arabidopsis and Thellungiella plants were grown hydroponically for 4 weeks as described in Chapter 2 (Section 2.2.1) before being subjected to salt treatment.

The treatments were applied by changing the growth solution to fresh MNS ('Control') or to MNS with additional 100 mM NaCl ('+NaCl'). Arabidopsis and Thellungiella plants were treated for 24 h at the same time. After treatment, root tissues were excised and flash frozen in liquid N_2, then the shoots were harvested in the same way. Frozen tissues were ground to a very fine powder in liquid N_2 with a mortar and pestle. The tissue powder was stored at – 80 °C until use. About 12 plants were pooled as a sample

in each replicate. Four different batches of plants were grown and harvested independently.

4.2.2 Preparation of total RNA samples

4.2.2.1 RNA isolation

RNeasy® Plant Mini Kit (Qiagen Ltd., West Sussex, UK) was used to isolate total RNA. Four columns were used to provide enough RNA for each sample. About 100 mg of tissue powder was applied to each column to obtain an optimal RNA yield and purity. RNA isolations were performed according to the RNeasy® protocol.

Briefly, for each column about 100 mg tissue powder was digested by 450 µl Buffer RLT (containing 4.5 µl β-Mercaptoethanol (β-ME)) in a centrifuge tube. The lysate was centrifuged at full speed for 2 min through the QIAshredder spin column into a 2 ml collection tube. The supernatant was mixed with 0.5 volume (usually 225µl) ethanol (96-100%) in a fresh tube. The mixture was centrifigated at ≥8000×g for 15 s through the RNeasy mini column, placed in a 2 ml collection tube (Rnase free). The RNeasy column was washed once with 700 µl Buffer RW1 and twice with 500 µl Buffer RPE. Finally, the total RNA was eluted twice from the RNeasy column with 30-50 µl Rnase-free water.

4.2.2.2 Agarose gel electrophoresis of the RNA

The quality of RNA samples was monitored by RNA agarose gel electrophoresis. An appropriate amount of agarose was added to the required volume of 1×TBE buffer (90 mM Tris-borate, 2 mM EDTA) to a final concentration of 0.5-2.0% (w/v) for gel casting. The agarose suspension was heated in a microwave oven until all the agarose dissolved completely. When the melted agarose solution had cooled down to about 60 °C, 10 mg/ ml ethidium bromide was added to a final concentration of 0.5 µg/ ml. The agarose

solution was then poured into the electrophoresis apparatus and allowed to set for 30 minutes at room temperature. After the gel had set, enough 1×TBE running buffer was added into the tank to submerge the gel to a depth of approximately 1-2 mm.

About 1 μg RNA from each sample containing a one-tenth volume of loading dye buffer (50% (v/v) glycerol, 1 mM EDTA (pH8.0), 0.25% (w/v) bromophenol blue), was loaded into the loading pockets. The electrophoresis was carried out at 100 volts (constant voltage) at room temperature until the bromophenol blue band had migrated down two-thirds of the gel.

4.2.2.3 Quantification of the RNA

The concentrations and purities of RNA samples were determined by spectrophotometry. Four μl of the isolated RNA sample were diluted to 100 μl with double distilled water. The diluted RNA samples were scanned between 200 and 340 nm. The absorbances at 260 nm and 280 nm were recorded. The ratio between absorbances at 260 nm (A260) and 280 nm (A280) was used to assess the purities of RNA samples. A ratio of 1.8-2 was considered acceptable. The concentration of RNA yield was calculated with the following equation:

$$\text{RNA concentration} = 40 \times A260 \times \text{dilution}$$

4.2.2.4 Ethanol precipitation of the RNA

About 1/10 volume of 3 M Na-acetate (pH5.5) and 2.5 volumes of absolute ethanol were added to the RNA sample and the RNA was precipitated at –20 °C for at least 1 h. The precipitate was spun down at ≥12,000 g at 4 °C for 20 min. The supernatant was removed and the pellet was washed twice with 0.5 ml 80% ethanol (stored at –20°C). Air dried pellets were resuspended in an appropriate volume with RNase-free water.

4.2.3 AMT microarray assay

Each array was hybridised with cDNA samples from control and treated plants labelled with the fluorescent dyes Cy3 and Cy5 respectively.

4.2.3.1 RNA preparation

One hundred micrograms of total RNA from each sample were used for each array. The RNA samples were concentrated with Microcon columns (Millipore Corporation, MA, USA) to 20 μl.

4.2.3.2 Reverse transcription

The concentrated RNA sample was mixed with 0.5 μl of 2 μg/μl Oligo dT_{20} primer. The mixture was incubated at 65 °C for 10 min, followed by 10 min at room temperature, then 2 min on ice. The mixture was combined with 16 μl labelling mastermix (see Table 4-2) and a 2 μl aliquot of the appropriate dye (Amersham CyDye Fluorescent Nucleotides, PA53021 and PA55021, Little Chalfont, UK). After warming at 42 °C for 2 min, 1.5 μl of Superscript II (200 U/μl) was added and the reaction mixture was incubated at 42 °C for 2 h. Another 1 μl of Superscript II was added half way through the incubation time. Once the dye had been added, the reaction mixture was protected from light. To terminate the reverse transcription, the reaction mixture was incubated with 10 μl 1 M NaOH at 65 °C for 10 min. Then 10 μl of 1 M HCl was added to balance the pH. Finally 200 μl of TE buffer (pH7.2) were added to the reverse transcription product before cleaning.

4.2.3.3 Cleaning of the labelled cDNA

The cDNA was cleaned with Qiaquick PCR purification kit (Qiagen Ltd., West Sussex, UK) according to the manufacturer's instruction. The total reverse transcription product, usually 260 μl, was mixed with 5 volumes (1300 μl) of buffer PB. The cDNA was

collected by spinning the entire sample-buffer mixture through a Qiaquick spin column. The column was washed with 750 µl of buffer PE. After spinning for 1 min, the column was transferred to a new collection tube. The clean cDNA was eluted with 50 µl buffer EB (10mM Tris-HCl, pH 8.5).

4.2.3.4 cDNA quantification and check of labelling

The amount of cDNA and the quality of the Cy-dye labelling were checked with spectrophotometry. Fifty µl of eluting solution (buffer EB) was used as blank. For each labelled cDNA samples (50µl), absorbance values at 230, 260, 280, 320, 550, 650 and 750 nm were recorded. The amount of cDNA and Cy3/Cy5 incorporation efficiencies were calculated according to the following formulas:

Amount of total cDNA in µg = $(OD_{260} - OD_{320}) \times 30 \times 50/1000$

Amount of Cy3 labelled cDNA in µg = $[(OD_{550}-OD_{650})/1.5\times10^5] \times 0.058 \times 50 \times 10^6$

Amount of Cy5 labelled cDNA in µg = $[(OD_{650}-OD_{750})/2.5\times10^5] \times 0.059 \times 50 \times 10^6$

Cy3/Cy5 incorporation efficiency = Amount of Cy3/Cy5 labelled cDNA/ Amount of total cDNA.

After spectrophotometry, samples were recovered, and vacuum dried for 30 min to less than 2 µl.

4.2.3.5 Array blocking

The arrays need to be blocked before hybridization. Thus, the array was incubated in pre-warmed blocking solution (5x SSC, 0.1% SDS and 1% (w/v) BSA) at 42°C for 45 min. After rinsing with deionized water for 5 times, the array was dried by spinning in a 50 ml tube at 3000 rpm for 10 min.

4.2.3.6 Hybridization

The dried cDNA samples were redissolved in 12 µl of preheated and well-dissolved formamide based hybridization buffer (MWG, Ebersberg, Germany). The two corresponding Cy3-/Cy5- labelled cDNA samples (root or shoot tissues from control and salt-treated plants of the same species) were combined and heated at 95°C for 3 min. After 30 sec on ice, the combined labelled cDNA was collected in the bottom of the tube by brief centrifugation and left at room temperature for several minutes before hybridization.

The labelled cDNA (target) was applied to the middle of the blocked array. The array was carefully covered with a cover slip so that no bubbles were trapped underneath. The array was locked in a hybridization chamber (MWG, Ebersberg, Germany) with 10 µl of water added in each corner of the chamber to maintain humidity. The hybridization chamber was incubated at 42°C in a water bath for 16- 24 h. During the incubation the chamber was protected from light with aluminium foil.

4.2.3.7 Washing

All washing buffers (Table 4-3) were pre-warmed at 30°C before washing. The cover slip was removed by gently shaken the array in washing buffer 1. During each washing step, the array slide was gently shaking in washing buffer at 30°C. After washing, the array was dried by spinning at 3000 rpm for 10 min. The hybridized array was stored in a dark, cool and dry place.

4.2.3.8 Array scanning

The array was scanned with a Packard™ ScanArray® Lite microarray scanner (Perkin Elmer Life Sciences, Monza, Italy) at wavelengths of 543 (Cy3) and 633 (Cy5) nm. Laser strength and gain were adjusted so that average signal intensities from both

channels were approximately similar. The array image was visualized and quantified with QuantArray® software.

4.2.4 Array analysis

4.2.4.1 Determination of expression levels

The extracted probe intensities from each sample and experiment were sorted in ascending order. All the sorted lists were arranged together in an EXCEL spreadsheet. The average intensity of the lowest 20 genes was used as background intensity. The genes with an intensity above twice the corresponding background intensity were considered as being expressed. The number of expressed genes was counted, and the percentage of expressed genes/ all genes was calculated for each gene list. Finally a representative percentage of expression was decided for each species by taking the biggest percentage of expression from either roots or shoots.

4.2.4.2 Signal distribution curve

The extracted probe intensity lists from the same tissue with the same treatment for each species were sorted in ascending order and arranged together in an EXCEL spreadsheet. The mean intensity was calculated for each probe. For example, all the three sets of signal intensities obtained from control Arabidopsis shoots were averaged after sorting. The list of average intensities from control Arabidopsis were sorted again and counted to collect the numbers of the genes whose intensity were above a certain intensity. A signal intensity distribution curve was drawn by plotting the numbers of the probes displaying an intensity above a given value, against this intensity value. In this way the raw data distribution for both shoots and roots from the two species was assessed.

4.2.4.3 Normalization

The extracted probe intensity profiles from different arrays were normalized with quantile normalization (Bolstad et al., 2003). For all experiments the probe intensities of each array channel were ranked in ascending order and arranged in an EXCEL spreadsheet. The intensity values for each rank position (quantile) were replaced with the average value of this quantile across all the entire data set. This process transformed the quantiles in each data set to the same value, thus resulting in identical distribution of the signal intensities. Note that after normalization each quantile has the same value but may correspond to a different gene for each sample. The normalized data sets were re-ordered and used for RP and iGA analysis.

4.2.4.4 Identification of differentially expressed genes

Differentially expressed genes were identified using the Rank Product method (RP, Breitling et al., 2004b). This is a non-parametric test that combines information on fold changes of expression with information on consistency of the data across replicate experiments. The probes (genes) were ranked according to their normalized expression ratio between each pair (fold changes). Sample pairs consisted in control and salt-treated tissue from the same species and the same experimental replicate. Two lists of these ratios were produced for each sample pair, ranked in either ascending or descending order. After each sorting process each gene had been assigned a rank in each sorted list. Ranks of each gene over all replicates were multiplied to obtain rank products. Finally, the gene list from each comparison was sorted according to RPs in ascending order. E-values were assigned to each RP by comparing the actual RP with the RP obtained for the same data after 100 random permutations. False discovery rates (FDR) were calculated by dividing the E-values by their position in the final ranked list.

An FDR of <1% means that 1% or less of the genes up to this position are expected to be observed by chance (false positives).

4.2.4.5 Identification of differentially expressed functional groups of genes

Genes were classified into membrane transporter families using the original annotation of the AMT array (Maathuis et al., 2003). Differentially expressed subsets of genes within a particular transporter family were identified using iterative Group Analysis (iGA, Breitling et al., 2004a). iGA determines the functional classes that are most enriched at the top of the gene lists sorted by rank products (separately for up- and down-regulation). The iGA procedure is based on calculating p-values using the hypergeometric distribution. For each functional class it iteratively finds the subset of members that minimizes this p-value. Only genes that were annotated in a specific classification scheme were considered for the analysis. As in the case of single genes, a multiple testing problem is encountered, because so many groups are examined simultaneously. To correct for this, FDR was used as a statistical measure. An approximate FDR was obtained from comparing p-values for each subset of genes to those obtained from 100 randomly permuted lists. The methods used for the analysis of the microarray are further discussed in the Discussion part of this chapter (4.4.1).

4.3 Results

4.3.1 Signal intensity and distribution

Typical overlay images of AMT arrays hybridized with cDNA from roots of Arabidopsis and Thellungiella respectively are shown in Figure 4-2. Although the overall signal intensity was lower with Thellungiella cDNA, most spots that produced a significant signal with Arabidopsis cDNA did so too with Thellungiella cDNA. In order to quantatively compare the signal levels produced by the membrane transporter

genome between Arabidopsis and Thellungiella, signal intensities of all the probes on each array were sorted in ascending order. The absolute signal intensities ranged between values of 200 and 55,000. A signal higher than two times of the background intensity, was considered to represent genes that were expressed. In Arabidopsis plants, an average of 73% of the membrane transporter genes represented on the array were expressed in either roots or shoots. In Thellungiella plants, 47% of all the probes produced a significant signal. This demonstrated suitability of the AMT array for cross-species gene expression analysis with Thellungiella mRNA sample.

The distribution of the signal intensities of all the probes obtained for a particular tissue and treatment were compared between Arabidopsis and Thellungiella (Figure 4-3). The signals were distributed over the entire intensity spectrum for both Arabidopsis and Thellungiella cDNA. In general, hybridization of the array with Thellungiella cDNA resulted in fewer probes producing high signals than hybridization with Arabidopsis cDNA. The lower signal intensity obtained with Thellungiella cDNA could be due to less efficient hybridization because of sequence differences between mRNAs and their (Arabidopsis) probes, or to lower expression levels of many transporter genes. Interestingly, the distribution curves were more similar between the roots of Arabidopsis and Thellungiella, indicating that only a small proportion of the differences between the signal intensities is actually due to sequence differences. Salt treatment increased the number of probes producing higher signals both for Arabidopsis and for Thellungiella cDNA, and it also increased the gap between the distribution curves of Arabidopsis and Thellungiella. This could be evidence for a salt-induced overall increase in (transporter) mRNA which was more pronounced in Arabidopsis than in Thellungiella.

4.3.2 Difference in gene expression levels of individual genes between Thellungiella and Arabidopsis

The expression levels of individual membrane transporter genes were directly compared between the two species after signal normalization (see Material and methods, section 4.2.4.3).

Differentially expressed genes were identified using RP (see section 4.2.4.4, Tables 4-4 and 4-6). Note that differences in the mRNA sequence between Thellungiella and Arabidopsis might lead to false identification of genes with lower (Table 4-6) but not higher expression in Thellungiella (Table 4-4).

Iterative group analysis (iGA) was applied to the ranked lists derived from RP analysis to identify transporters within functional categories that were, as a group, differentially expressed between the two species (Tables 4-5 and 4-7).

4.3.2.1 Transcripts that are more abundant in Thellungiella than in Arabidopsis

Table 4-4 presents membrane transporter genes with higher mRNA levels in Thellungiella than in Arabidopsis under control condition (column 3) and/or after 24 h treatment with 100 mM NaCl (column 4). A '+' indicates that differential expression of the gene was detected above the significance cut-off under control or '+NaCl' conditions. The cut-off FDR values were chosen as 10% for the shoot data and 1% for the root data due to a much higher numbers of differentially expressed genes in the roots than in the shoots. The genes were arranged according to functional groups. Functional groups of transcripts that were identified by iGA, as being more abundantly expressed in Thellungiella than in Arabidopsis are presented in Table 4-5. The significance cut-off was chosen at $P < 0.015$ (see Material and methods, section 4.2.4.5).

- V-type pump and PPase

Under control conditions, in the roots thirteen subunits of the vacuolar H-ATPase and a PPase gene showed higher mRNA levels in Thellungiella than in Arabidopsis. The difference in V-type pump and PPase expression between the two species persisted after 24 h treatment with 100 mM NaCl. iGA analysis confirmed that vacuolar proton pump subunits and PPases as functional groups were expressed more abundantly in the roots of Thellungiella than in Arabidopsis both with and without salt treatment.

- Ion transporters for inorganic nutrients transport

A number of transcripts for transporters that are responsible for inorganic nutrient uptake are more abundant in the roots of Thellungiella than in the roots of Arabidopsis, including a nitrate transporter (NRT2,6), an ammonium transporter (AMT1,3), a phosphate transporter (PHT1,5) and metal transporters (COPT3, IRT1 and ATMTP1). The expression differences in these genes between the two species were found both with and without salt treatment. Nitrate transporters were also identified as a group by iGA analysis as being expressed more abundantly in Thellungiella than in Arabidopsis, in both roots and shoots.

In the shoots, inorganic nutrient transporters were the majority of the genes that were expressed at higher levels in Thellungiella than Arabidopsis. For example, the expression level of a putative high affinity K transporter, KUP/HAK/KT8, in the shoots of Thellungiella was higher than the expression level of its analogue in Arabidopsis. The expression difference of this putative K transporter between the two species was the strongest of all identified differences in the membrane transporter transcriptome. A putative cation-H antiporter (CHX8), a putative Na-H antiporter (NHX3), a Mg transporter (MGT1), a putative nitrate transporter (NAR2-like1) and a putative sulphate transporter (SULTR3.3/AST91) were also expressed at higher levels in the shoots of Thellungiella than Arabidopsis. After salt treatment, the expression difference in the

nitrate and sulphate transporters between Arabidopsis and Thellungiella was no longer significant.

- Transporters for organic molecules

Transporters for organic molecules such as sugars, amino acid and peptides were expressed at higher levels in the roots of Thellungiella than Arabidopsis. These included two aminoacid transporters, ATENT7 and ATPROT1. Sugar transporters were identified by iGA as a group with higher expression levels in the roots of Thellungiella compared to Arabidopsis after salt treatment. Two aminoacid transporters were also detected to have higher mRNA levels in the shoots of Thellungiella than in the shoots of Arabidopsis.

- ABC transporters and auxin transporters

ABC transporters, mainly of MRP and PDR subfamily, were expressed at higher levels in the roots of Thellungiella plants than in Arabidopsis. The difference in expression levels between the two species was independent of salt treatment. Two MRP type ABC transporters were detected to have higher mRNA abundance in the shoots of Thellungiella than in Arabidopsis. After salt treatment a WBC type ABC transporter also appeared more abundantly in the shoots of Thellungiella than in Arabidopsis. However ABC transporters were not identified by iGA as a functional group differentially expressed between Arabidopsis and Thellungiella.

Two auxin transporters (ATAUXR2 and ATAUXR3) were expressed at higher level in the roots of Thellungiella compared with Arabidopsis under control condition. After salt treatment, only the difference in transcript level of ATAUXR3 was maintained. Nevertheless, auxin transporters was identified by iGA as a functional group with higher expression level in the roots of Thellungiella than in Arabidopsis ($P < 0.015$).

The differential expression of the auxin transporter group between the two species persisted when the plants were subjected to salt treatment.

- Other transporters

Two plasma membrane aquaporins were detected to have higher mRNA abundances in the shoots of Thellungiella than Arabidopsis. After salt treatment the expression difference of aquaporin PIP2,4 disappeared.

An unknown protein with 8 predicted transmembrane domains was expressed at higher level in the shoots of Thellungiella than in Arabidopsis. A transcript belonging to the secretory carrier family was also more abundant in the shoots of Thellungiella than in Arabidopsis, but only after salt treatment.

The functional groups identified by iGA whose expression levels in the shoots of Thellungiella are higher than in Arabidopsis included aquaporins, glutamate receptors and a small gene family SCAMP, possibly involved in membrane trafficking. The expression difference of these groups between the two species did not change with salt treatment.

4.3.2.2 Transcripts that produced lower hybridization signals in Thellungiella than Arabidopsis

Because cDNA from Thellungiella plants was hybridized to probes for Arabidopsis genes, it is not sure whether a Thellungiella/Arabidopsis intensity ratio smaller than 1 is due to lower expression levels in Thellungiella, or to a low homology of the Thellungiella gene to the corresponding Arabidopsis gene. Sequencing of the probe-corresponding regions of the Thellungiella genes would be necessary to distinguish between these two possibilities. Nevertheless, Table 4-6 presents the genes that produced a lower hybridization signal in Thellungiella than in Arabidopsis. Functional

groups with relatively lower signal intensity in Thellungiella than Arabidopsis identified by iGA are presented in Table 4-7.

- Calcium pumps and other P-type pumps

In the roots transcripts encoding all calcium ATPases except for ACA5, and a few other P-type pumps e.g. HMA5, HMA2 and PAA1 were detected with lower signal intensity when hybridized with cDNA from Thellungiella plants than when hybridized with cDNA from Arabidopsis plants grown under control condition. After salt treatment the signal difference in ACA3 between Arabidopsis and Thellungiella disappeared. Calcium transporters such as ACAs, calcium-H antiporters and putative calcium channels were all identified by iGA as functional groups that showed lower signal levels with Thellungiella cDNA than with Arabidopsis cDNA.

- Cation transporters

Three putative magnesium transporters (MGT4, MGT7 and MGT10) and a putative cation-proton antiporter (CHX26) showed lower signal intensity after hybridization with cDNA from the roots of Thellungiella than after hybridization with cDNA from Arabidopsis roots before salt treatment. After salt treatment lower signals of two more putative magnesium transporters (MGT8 and MGT9), three other putative CHXs (CHX15, CHX21 and CHX23) and a putative Na-H antiporter (NHX8) were detected for Thellungiella. As expected, magnesium transporters and CHXs, together with several other cation transporter groups such as Na-H antiporters, K efflux systems, K transporters and CNGCs, were identified by iGA as functional groups that had produced lower signal levels with Thellungiella cDNA compared to Arabidopsis cDNA ($P < 0.015$).

- Aquaporins

Aquaporins, mainly NIPs with 2 NLMs, 2 TIPs and a SIP, showed weaker signals on arrays hybridized with cDNA from the roots of Thellungiella than on those hybridized with cDNA from the roots of Arabidopsis. A few PIPs (PIP1,3, PIP2,1, PIP2,6) and TIP1,1 also showed lower signals with cDNA from the shoots of Thellungiella. These differences in aquaporin signals between the two species were not affected by salt treatment. Aquaporins were identified by iGA as a functional group producing lower signals with Thellungiella cDNA than with Arabidopsis cDNA both from roots and shoots ($P < 0.015$).

- ABC transporters

ABC transporters, mainly of the NAP and WBC subfamily, gave lower signals on the arrays hybridized with Thellungiella root cDNA than on those hybridized with Arabidopsis cDNA. These differences persisted after salt treatment. For the shoots, ATPDR8 and ATWBC12 produced lower signals with Thellungiella cDNA than with Arabidopsis cDNA. One more ABC transporter (ATATH1) was included in this group after salt stress.

- Transporters for organic molecules

A putative sugar transporter (STP38), a putative peptide transporter (PTR36) and an aminoacid transporter (At4g38250) were found to produce lower signals on the arrays hybridized with cDNA from the shoots of Thellungiella compared with those hybridized with cDNA from Arabidopsis shoots. After salt treatment, such differences appeared also for another sugar transporter (SUGAR2) and a peptide transporter (PTR15).

- Other putative transporters

Two members of the NST-TPT family (At4g09810 and At1g76670) and one member of the MC family (At4g39460) produced lower signals with cDNA from the shoots of

Thellungiella compared to Arabidopsis. Two MATE family members (At5g65380 and At1g12950) and a MPT family member joined this group after salt stress.

A few unknown membrane proteins with 6 or 10 predicted transmembrane spanning domains showed lower signal on the arrays hybridized with cDNA from the shoots of Thellungiella compared with those hybridized with cDNA from Arabidopsis shoots both before and after salt treatment (FDR < 10%).

iGA analysis of the root data recognized sulphate transporters and glutamate receptors as functional groups that gave lower signals with Thellungiella cDNA than Arabidopsis cDNA ($P < 0.015$). For the shoots, functional groups with lower signals from Thellungiella cDNA than Arabidopsis cDNA also included aminoacid transporters, CNGCs and the NST-TPT family.

- Known stress induced genes

Two known stress induced genes (At1g57550 and At2g24040) gave lower signals on the arrays hybridized with Thellungiella root cDNA than on those with Arabidopsis root cDNA. Known cold or drought induced genes such as COR78, KIN2 and LTI6A showed considerably lower signal intensity with cDNA from Thellungiella shoots than with cDNA from Arabidopsis shoots. The signal differences of these genes between the two species persisted after salt treatment. So-called 'stress induced genes' were identified as a group by iGA analysis that gave lower signal levels when hybridized with Thellungiella shoots cDNA.

4.3.3 Salt induced changes in gene expression

4.3.3.1 Transcripts commonly regulated by salt stress in the two species

RP and iGA methods were used to analyse responses of the membrane transporter transcriptome to salt in Arabidopsis and Thellungiella. In general, more genes were regulated by the same salt treatment, 100 mM NaCl for 24 h, in Arabidopsis than in

Thellungiella. Nevertheless, many of the salt-regulated genes responded in both species. The fifty most significantly up- and down-regulated genes of either Arabidopsis or Thellungiella were checked against the list of the top one hundred transcripts regulated in the same direction in the other species. In the roots, about 50% of the top 50 salt regulated genes of both species showed similar regulation in response to salt in the other species. In the shoots, the overlap was much lower (about 20%). Furthermore, there were more common transcripts among the down-regulated than among the up-regulated genes (Figure 4-4). No up-regulated gene was found with an FDR < 10% in the shoots of Thellungiella. Within up to 30% FDR only one gene was commonly induced by salt in the shoots of both species (Table 4-8), which is the low-temperature stress induced gene COR78 (Figure 4-5). Genes that were commonly regulated by salt in both Arabidopsis and Thellungiella within 30% FDR are shown in Figure 4-5. The known stress-induced genes KIN2 and P5CS and a putative sugar transporter (STP13) were commonly induced by salt in the roots of both Arabidopsis and Thellungiella. Aquaporins, mostly TIPs, and a putative peptide transporter (PTR44) were commonly down-regulated by salt in the roots of both species. In the shoots, aminoacid transporters (ATLHT1 and ATPROT1), a peptide transporter (PTR7) and an ABC transporter (ATMRP9) were down-regulated by salt in both species.

4.3.3.2 Salt induced changes of gene expression that are specific for Arabidopsis

- Up-regulated genes in the roots

In the roots of Arabidopsis, salt stress induced the expression of many putative transporters including a putative calcium pump (ACA13), a putative peptide transporter PTR35, a few ABC transporters mainly of the MDR subfamily, and two members of the MATE family but not Thellungiella (Table 4-10). Two unknown membrane proteins with 6 predicted transmembrane spanning domains (AT4g37030 and AT5g35735) were

also found to be up-regulated by salt in the roots of Arabidopsis (Table 4-10). No membrane transporters responsible for inorganic nutrient uptake were significantly up-regulated by salt treatment in the roots of Arabidopsis alone. Nevertheless, phosphate transporters were identified by iGA as a functional group that is up-regulated by salt stress in the roots of Arabidopsis (Table 4-13). The MATE family and the FBT family were also identified by iGA to be up-regulated by salt in the roots of Arabidopsis ($P < 0.015$).

- Down-regulated genes in the roots

In addition to those aquaporins that were commonly regulated in Arabidopsis and Thellungiella, other genes of this family were only regulated in Arabidopsis (e.g. PIP2,3, PIP2,4, NIP1,1, Table 4-12). The only cation transporter that was down-regulated specifically in the roots of Arabidopsis after salt treatment was a putative metal transporter, ATMTPA1. An aminoacid transporter (ATAAP2) and a glutamate receptor GLR 2.3 were also found to be down-regulated by salt in the roots of Arabidopsis. Functional groups that were down-regulated by salt in the roots of Arabidopsis according to iGA included aquaporins, vacuolar H-ATPase, sulphate transporters, Cl channels and the MPT family ($P < 0.015$, Table 4-13).

- Up-regulated genes in the shoots

In the shoots of Arabidopsis, salt stress specifically induced the expression of cation transporters such as the putative K transporter, KUP/HAK/KT6 and the putative Ca-H antiporter, CAX3, as well as anion transporters such as a putative nitrate transporter (NAR2-like1), a putative sulphate transporters (SULTR3.1/AST12) and a putative Na-sulphate co-transporter (At5g47560) (Table 4-9). Several putative aminoacid transporters (At2g41190, At5g65990 and At1g58360) and a putative peptide transporter (PTR15) were up-regulated by salt treatment in the shoots of Arabidopsis but not

Thellungiella. Two ABC transporters (ATWBC26 and ATPDR1), two MATE family members (At5g65380 and At4g39030) and three unknown membrane proteins with 6 (At3g20300) or 7 predicted transmembrane spanning domains (At1g78610 and At4g21570) showed the same response. iGA identified sulphate transporters, Ca-H antiporters, the FBT family and the MFS family as groups that have increased transcript abundance in the shoots of Arabidopsis after salt treatment ($P<0.015$, Table 4-13).

- Down-regulated genes in the shoots

In the shoots of Arabidopsis more genes were down-regulated after salt treatment than up-regulated. Aquaporins, especially tonoplastic aquaporins (TIPs) featured strongly among the down-regulated transcripts specific for this species (Table 4-11). The high affinity K transporter HAK5 was down-regulated by salt stress in the shoots of Arabidopsis, and so was a member of the CNGC family (CNGC8), a putative cation-H antiporter (CHX15) and the P-type H-ATPase, AHA2. Reduced levels of transcript after salt treatment were also detected for a putative Cl channel (PORIN3), a putative peptide transporter (PTR20) and several aminoacid transporters including AAP6 and AAP10. Salt treatment also inhibited specifically in Arabidopsis the expression of two MDR type ABC transporters (ATMDR11 and ATMDR18) and an unknown membrane proteins with 6 predicted transmembrane spanning domains (At4g12980). Aquaporins, aminoacid transporters and putative anion exchangers were identified by iGA as functional groups that are down-regulated by salt in the shoots of Arabidopsis ($P < 0.015$, Table 4-13).

4.3.3.3 Salt induced changes of gene expression that are specific for Thellungiella

ADH1 and an unknown membrane protein with 7 predicted transmembrane spanning domains (At1g12730) were up-regulated specifically in the roots of Thellungiella (Table

4-10). The MATE family was identified as a group that are up-regulated in the roots of Thellungiella after salt stress by iGA analysis ($P < 0.015$, Table 4-13).

Several cation channels were down-regulated after salt treatment in the roots of Thellungiella including CNGC5, CNGC8 and a putative metal transporter (ZIP8) (Table 4-12). A putative malate transporter (At5g64280) showed also lower transcript abundance after salt treatment in the roots of Thellungiella (Table 4-12). Functional groups down-regulated by salt in the roots of Thellungiella are quite diverse (Table 4-13). Aquaporins and both P-type and V-type pumps as functional groups were down-regulated by salt in the roots of Thellungiella ($P < 0.015$). Other functional groups down-regulated in the roots of Thellungiella after salt treatment include CNGCs and K channels, as well as genes annotated to be aminoacid, metal or anion transporters ($P < 0.015$).

There were no individual genes that was significantly (FDR < 10%) up-regulated in the shoots of Thellungiella. However, the secretory carrier family (SCAMPs), stress induced proteins, the inner mitochondria membrane protein family and auxin transporters were up-regulated as functional groups after salt treatment in the shoots of Thellungiella ($P < 0.015$, Table 4-13).

A putative ABC transporter (ATMRP8) and a putative aminoacid transporter (At2g39130) were specifically down-regulated by salt in the shoots of Thellungiella (Table 4-11). Only aminoacid transporters were identified as a functional group by iGA that was down-regulated by salt ($P < 0.015$, Table 4-13).

4.4 Discussion

4.4.1 Employing microarrays for the comparitive study of gene expression in Thellungiella and Arabidopsis

Microarrays are the most efficient method to measure transcript levels of a large number of genes. If, as in the case of Thellungiella, the genome of the studied organism has not been sequenced, microarray technology can be employed in two ways: either new microarrays are prepared from an EST cDNA library of this organism, or existing arrays for a closely related species are used. Both approaches have been applied to Thellungiella.

Wong et al. (2006) prepared a cDNA microarray using sequences derived from stress-induced cDNA libraries of the Yukon ecotype of *Thellungiella halophila* (Wong et al., 2005), whereas Volkov et al. (2004), Taji et al. (2004), Gong et al. (2005) used various types of Arabidopsis arrays. The disadvantage of the first approach is that the microarray analysis is limited to those transcripts that are present in the library (e.g. the current Thellungiella array contains only 3628 probes). The advantage is that the array may contain probes for genes that are specific for Thellungiella.

When using arrays from closely related species a compromise has to be found between achieving a good hybridization signal even if probe and target sequences are not 100% identical and avoiding cross-hybridization between highly homologous members of gene families. Arabidopsis arrays based on long cDNA sequences as used by Taji et al. (2004) are likely to produce mixed signals from closely related Thellungiella genes, whereas arrays with very short probe sequences (e.g. Affymetrix) produce very low hybridization signals when hybridized with Thellungiella cDNA (21%, Volkov et al., 2004). Gong et al. (2005) employed an Arabidopsis array based on the 70-mer probes of the Qiagen-Operon Arabidopsis Genome Array Ready Oligo Set (AROS) (Arizona

array, http://ag.arizona.edu/microarray/) and achieved hybridization signals of 60% with Thellungiella shoot RNA samples compared to 80% with Arabidopsis shoot RNA samples.

In this study I employed an Arabidopsis Membrane Transporter array which is based on 50-mer probes. The average signal intensity obtained with Thellungiella shoot RNA samples was 47% (47% for root RNA samples) compared to 63% obtained with RNA samples from Arabidopsis shoots (73% for root samples). The apparent expression levels are comparable to the levels reported by Gong et al. (2005), although the difference between the two species was slightly bigger. One possible explanation for the bigger inter-species signal difference on the AMT-array could be that sequence homology between the two species is lower among membrane transporters than at the whole genome scale. Another possibility is that the stringency of the washing was different in the two studies. However, I tested various stringencies of the wash solutions (e.g. buffers containing 2×, 1×, or 0.5× SSC) and found that signal intensities decreased more or less in parallel for the two species. The general good hybridization signal obtained with 50-70 mer probes reflects the high genetic similarity of the two species despite their differences in stress tolerance.

Indeed, comparison of EST sequences from Arabidopsis and Thellungiella revealed high sequence similarity for the majority of transcripts. For example, most Thellungiella transcripts for well-known housekeeping genes in photosynthesis and basal metabolism showed between 90% and 95% identity at the nucleotide level, indicating that they are orthologues to Arabidopsis genes (Inan et al., 2004). However some other transcripts of Thellungiella, many in categories related to stress responses, showed significantly lower identity scores with Arabidopsis genes at the nucleotide level (Inan et al., 2004). Wang et al. (2004) sequenced more than 1500 randomly selected clones from a NaCl-treated

cDNA library of Thellungiella. They reported the identity between Thellungiella and Arabidopsis cDNA sequences to be 95.76% among all ESTs and 95.36% for non-redundant clones (Wang et al., 2004). Another analysis covering 6578 ESTs from the Yukon ecotype of Thellungiella reported that 94.1% unigenes encoded products that were highly similar to Arabidopsis in their amino acid sequence and only 1.5% had no match within the Brassica family (Wong et al., 2005).

Despite the high homology between Arabidopsis and Thellungiella genes and despite the fact that Thellungiella mRNA produces an average a good signal intensity on Arabidopsis 50-70 mer arrays, the inter-species comparison of expression levels of individual transcripts is still accompanied by some uncertainty. If a probe produces a lower signal when hybridized with Thellungiella mRNA than when hybridized with Arabidopsis mRNA, there are several possible reasons: 1. the respective gene has a lower transcript level in Thellungiella than in Arabidopsis, 2. the Thellungiella gene has low homology to its Arabidopsis analogue in the sequence of the probe region, 3. the Thellungiella gene has low homology to its Arabidopsis analogue in the overall gene sequence, 4. the gene is absent in the Thellungiella genome. A clear distinction between these possible reasons can only be obtained through sequencing. By contrast, if a probe produces a higher signal with Thellungiella than with Arabidopsis mRNA the only possible explanation is that the respective transcript is indeed more abundant in Thellungiella than in Arabidopsis. All gene expression studies using Arabidopsis arrays face the common problem that genes unique to Thellungiella cannot be analyzed.

4.4.2 Experimental design and data analysis

This study assessed differences in transcript levels between two different species (Arabidopsis and Thellungiella) and between two different conditions (with and without salt). Since no more than two samples can be analysed on the same array, and I decided

to hybridize control and salt-treated samples on the same array, the comparison between species was carried out using data from different arrays.

To make such comparison reliable, a good normalization method is crucial. Quantile normalization was identified as the best available option (Bolstad et al., 2003) and applied in this study (see Material and Methods, section 4.2.4.3). This method replaces lists of ranked signal intensities derived from individual samples with lists of average signal intensities for each rank. Thus, equal rank positions in the normalized lists have equal intensity value (but are not necessarily occupied by the same genes). Because quantile normalization adjusts the signal intensity distribution between the different arrays (or array channels), it is important to verify prior to normalization that the raw signal intensities are relatively evenly distributed over the entire signal range. This was a particular concern with Thellungiella samples. As shown in Figure 4-3 it appears that even with the heterologous RNA samples a probe length of 50 bp is long enough to produce a continuous spectrum of signal intensities over a wide range of signal intensity. The normalized data were subsequently used to calculate for all genes the ratios between their expression levels in Arabidopsis and Thellungiella, and between their expression levels in plants exposed to low or high salinity.

RP and iGA were applied to the normalized data. Most data-mining methods for microarray data only focus either only on fold-changes or on statistical significance. RP combines information on fold-changes with information on the consistency of such changes in biological replicates. Therefore this method can extract meaningful information from data sets that are characterised by relatively small fold changes, as in the case for membrane transporters. iGA considers transcriptional regulation of groups of genes rather than individual genes. This method is motivated by the notion that common regulation of a number of functionally related genes could achieve a

physiological response, even if the expression change of each individual gene is relatively small. In contrast to other methods e.g. determination of the percentage of the number of genes belonging to a functional group in a pre-defined list of 'differentially regulated' genes, iGA is not based on cut-offs. Instead, the algorithm 'walks down' the entire list of ranked genes and identifies subsets of genes within functional groups for which the probability to appear together relatively high up the list is very small (p-values in tables 4-5, 4-7, 4-13). This method is particularly suitable to extract physiologically relevant information from poorly replicated microarray data as members of a particular functional group or gene family serve as 'internal replicates'. However, this method will not identify 'functional modems' that are constructed from members of very different gene families. These can only be identified through cluster analysis based on a much larger number of conditions than applied in this study.

4.4.3 Differences in transcript abundance between Arabidopsis and Thellungiella

4.4.3.1 Known stress-inducible genes

A number of known stress-inducible genes were included as controls on the AMT array. These included pyrroline-5-carboxylate synthase (P5CS), a cold- and ABA- inducible gene (KIN2), a cold regulated gene COR78 and low temperature inducible genes (LTI family). The first plant P5CS gene was cloned from mothbean. It has both gamma-glutamyl kinase and glutamic-gamma-semialdehyde dehydrogenase activities that catalyze the first two steps in proline biosynthesis (Hu et al., 1992). Proline is known to be accumulated by plants as compatible osmoticum during osmotic stress (Keller et al., 1973; Serrano, 1996). Expression of P5CS transcripts is also induced by salt stress in Arabidopsis (Yoshiba et al., 1995), rice (Igarashi et al., 1997) and alfalfa (Ginzberg et al., 1998). KIN2 belongs to a family of two cold-inducible genes in Arabidopsis, and is

induced by ABA treatment, drought and salt stress (Kurkela and Borg-Franck, 1992). COR78 or RD29A was first identified as a cold responsive gene (Nordin et al., 1993). It has at least two cis-acting elements involved in the ABA-associated response to dehydration and the sensing of changes in osmotic potential (Yamaguchi-Shinozaki and Shinozaki, 1994). The cis-acting DRE (dehydration-responsive element) of the COR78 promoter is involved in the response to dehydration, high salt, and low temperature but does not function in the ABA signalling pathway (Yamaguchi-Shinozaki and Shinozaki, 1994). LTI2A and LTI2B were identified by (Jarillo et al., 1994) as so-called Rare Cold- Inducible cDNAs, which are similar to 14-3-3 proteins involved in the regulation of multifunctional protein kinases. Expression of the LTI2 genes was induced by low temperature, ABA and dehydration but did not respond to salt and anaerobiosis treatment (Capel et al., 1997).

Taji et al. (2004) found that a large number of genes that are inducible by abiotic- and biotic stress in Arabidopsis, i.e. P5CS, were expressed in Thellungiella at higher levels than in Arabidopsis even in the absence of stress. However these findings were not confirmed in my microarray experiments. No difference in expression levels of P5CS between Arabidopsis and Thellungiella was found. COR78, KIN2 and LTI6A were even expressed at lower levels in the shoots of Thellungiella than in Arabidopsis both under control and high salt conditions. Two homologues of LTI6A (At1g57550 and At2g24040) were expressed at higher levels in the roots of Arabidopsis than in Thellungiella.

Thellungiella is able to resist multiple abiotic stresses including salt, drought and low temperature stresses (Bressan et al., 2001; Zhu, 2001; Wong et al., 2005). It requires stronger stress to show damage symptoms compared with Arabidopsis. Therefore if the expression of stress-induced genes is linked to the appearance of stress symptoms, these

genes might require stronger stress to be induced in Thellungiella to the same extent as in Arabidopsis.

4.4.3.2 Proton pumps

One of the most striking difference of constitutive expression differences between Thellungiella and Arabidopsis revealed in this study is the higher levels of transcripts for most subunits of vacuolar H-ATPase in the roots of Thellungiella. These concern both soluble V1 and membrane anchoring V0 sectors. One gene encoding a vacuolar PPase (AVP1) was also expressed at higher levels in Thellungiella than in Arabidopsis both with and without salt stress. Vacuolar pumps are known to be transcriptionally activated by salt stress in several plant species (Dietz et al., 2001; Wang et al., 2001). Maathuis et al. (2003) reported that most subunits of the vacuolar H^+ ATPase were up-regulated in response to 80 mM NaCl in the roots of Arabidopsis. Up-regulation of vacuolar H-ATPase subunits and the PPase was also found in the facultative halophyte *Mesembryanthamum crystallinum* (Kluge et al., 2003) and in *Suaeda salsa* (Guo et al., 2006).

Maintaining an electrochemical gradient for protons across tonoplast membrane is critical for plant ion homeostasis in general as it provides the driving force to store nutrients such as K and Ca in the vacuole through the action of H-cation antiporters. Under salt stress trans-tonoplast H pumping is particularly important as it allows the plant to remove Na ions from the cytosol via Na-H antiport systems. Indeed over-expression of the PPase in Arabidopsis leads to enhanced salt tolerance (Gaxiola et al., 1999; Guo et al., 2006).

Furthermore, expression of vacuolar proton pumps might support the enlargement of vacuolar lumen required to accommodate the incoming Na. Mimura et al. (2003) reported rapid increase in the vacuolar volume and activation of the tonoplast ATPase

and PPase in response to salt in salt-tolerant mangrove and barley cells. The authors suggested that part of the activation of the vacuolar enzymes could be required for vacuole synthesis itself (Mimura et al., 2003).

Constitutively high expression levels of vacuolar proton pumps in Thellungiella might reflect the fact that this species is adapted to an environment where high salinity is either a permanent feature or occurs periodically and rapidly.

Establishment of an electrochemical gradient across the plasma membrane is essential for nutrient acquisition and export of Na. Uptake of sulphate, phosphate, nitrate, amino acids and sugar occurs usually via H^+- coupled symporter systems (Boorer and Fischer, 1997; Forde, 2000; Vitart et al., 2001; Yildiz et al., 1994; Zhou et al., 1997). Proton-coupled antiport is required to actively export Na from the cytoplasm into the apoplast. Maathuis et al. (2003) found an increase in transcript level of the plasma membrane H^+-ATPase, AHA2, in the roots of Arabidopsis after a 24 h treatment with 80 mM NaCl. Therefore AHA2, which is mainly expressed in the root epidermis, could play an important role in providing the electrochemical H gradient for active Na export. In Thellungiella, AHA2 was expressed constitutively at higher levels than in Arabidopsis, but this difference was only significant in the shoots. Interestingly, a hitherto uncharacterised putative Na/H antiporter, NHX3, mirrored AHA2 expression, both in Arabidopsis and in Thellungiella.

4.4.3.3 Ca pumps and divalent cation transporters

Many genes encoding Ca pumps and divalent cation transporters were found to produce lower hybridization signals with Thellungiella cDNA than with Arabidopsis cDNA. The list of genes showing the most significant difference contains Ca pumps (ACA family), Mg transporters (MGT family), and putative cation-proton antiporters (CHX family); and iGA pointed to Ca-H antiporters (CAX) and putative Ca channels as having lower

expression levels in Thellungiella than in Arabidopsis. Ca-pumps and CAXs are important for maintaining a low cytosolic Ca concentration (Reddy, 2001; Sanders et al., 2002) The CHX transporters belong to the CPA2 family of the cation/proton antiporters. Only a few isoforms of CHX family have been functionally characterized; e.g. AtCHX17 affects K homeostasis (Cellier et al., 2004), AtCHX21 encodes for a putative Na transporter (Hall et al., 2006) and AtCH23 controls chloroplast pH (Song et al., 2004).

The most conspicuous difference was found for members of the ACA family of Ca pumps with 12 out of 13 ACA genes producing a significantly lower signal in Thellungiella roots than in Arabidopsis roots. As pointed out before this could be due to lower expression levels or to low sequence similarity (at least) in the probe region. If the latter is the case the signal difference should appear in both roots and shoots as long as the gene is expressed in both tissues. Most isoforms of the Ca pumps were expressed in the roots of Thellungiella with signals above 1000, except for ACA9, ACA12 and ACA13 which produced signals of about double the average background value. Only ACA7 showed no sign of being expressed in the roots of Thellungiella (signal intensity similar to background value). In the shoots of Thellungiella, fewer Ca pumps were expressed. ACA1, ACA2 and ACA10 gave signals above 1000, and signals of ACA2, ACA8 and ACA11 were about double the average background value. None of these six ACA genes showed a significant difference in hybridization signals between Thellungiella and Arabidopsis in the shoots, indicating that low sequence similarity is probably not the reason why these genes produce lower signals in the roots of Thellungiella than in the roots of Arabidopsis.

It is therefore likely that the lower signal differences indicate true differences in transcript levels of root Ca pumps between Arabidopsis and Thellungiella. Interestingly,

measurements of ion concentrations showed lower overall accumulation of Ca and Mg in Thellungiella compared to Arabidopsis (Figure 3-1). Thus root cells might experience less pressure to actively export Ca from the cytoplasm and therefore require fewer Ca pumps. Lower expression of gene encoding Mg transporters and CHX-type transporters in the roots of Thellungiella might also reflect a lower requirement for compartmentation of Ca and Mg, and if any of these genes is involved in uptake of divalent cation, their differential expression could even be the reason for the observed species-specific ion profiles (Figure 3-1).

4.4.3.4 Monovalent cation transporters

A putative high affinity K transporter, KUP/HAK/KT8, and a hitherto uncharacterised Na/H antiporter NHX3, were expressed in the shoots of Thellungiella at significantly higher levels than in Arabidopsis. The KUP/HAK/KT family is named after their homology to bacterial K uptake permease (KUP; (Schleyer and Bakker, 1993)) and fungal high-affinity K transporters (HAK; (Banuelos et al., 1995)). The 13 members of this family in Arabidopsis are divided into four groups on a phylogenetic tree (Rubio, 2000). The major role of KUP/HAK transporters is in both high- and low-affinity K uptake (Véry and Sentenac, 2003), and it is possible that some isoforms (i.e. HAK5) might be involved in K/Na homeostasis during salt stress (Wang and Amtmann, unpublished results). Na-H antiporters function in the active export of Na from the cytoplasm and in its compartmentation (Apse et al., 1999; Shi et al., 2000).

It is possible that high transcript levels of these two genes reflect the adaptation of Thellungiella to a permanent or frequent high-salt environment. Even if Na uptake is restricted (see Chapter 2), leaf cells will experience apoplastic solutions that are high in Na under salt stress (e.g. high Na/K ratios) and KUP/HAK and NHX transporters will be required to support maintenance of cytoplasmic Na/K ratios. By contrast, the plasma

membrane Na/H antiporter, SOS1, for which constitutively high expression levels in Thellungiella, had been reported by Taji el al. (2004), did not produce a species-specific expression pattern in this study. One possible reason for the difference could be that the full length cDNA probes used by Taji et al. (2004) recognize not only the target genes but also other isoforms of the same gene family. For example, it is possible that the probe for SOS1 on the full-length cDNA array cross-hybridizes with NHX3 mRNA which in my study showed high expression levels in Thellungiella shoots (see above).

In addition to individual genes that showed highly significant differences between Thellungiella and Arabidopsis, the iterative group analysis identified subsets of genes belonging to K transporters, K efflux systems, metal transporters, CHXs and CNGCs. Plant CNGCs have a predicted structure of six transmembrane domains with a pore domain (P loop) between S5 and S6, and cyclic-nucleotide-binding (CNB) and CaM-binding (CaMB) domains overlapping each other in the C-terminus. The CNGC family has 20 members in Arabidopsis. Distinctive ion selectivity of the pore region and interference between CaMB and cyclic nucleotide binding activity set the difference between plant CNGCs and animal CNGCs as well as K-selective channels (Arazi et al., 2000; Talke et al., 2003). Although certain members of the CNGC family are potentially pathways for a voltage-independent inward current of Na in Arabidopsis (Maathuis and Sanders, 2001), heterologous expression studies suggest that neither AtCNGC1 are AtCNGC2 is responsible for this Na current (Hua et al., 2003; Leng et al., 2002; Leng et al., 1999).

4.4.3.5 Nitrate transport and N homeostasis

It was found that Thellungiella over-accumulates proline under conditions of both high and low salt (Inan et al., 2004 and Taji et al., 2004). Proline is an important compatible osmolyte that is used to balance the osmotic potential under salt stress (Adams et al.,

1998). Proline is synthesized *de novo* from glutamate (Hasegawa et al., 2000; Nanjo et al., 1999) leading to an increased nitrogen requirement of salt-stressed plants. In addition, competition of increased concentrations of chloride with nitrate uptake and accumulation has been hypothesized (Liu and Shelp, 1996). Therefore control of N homeostasis by salt-dependent regulation of nitrate and amino acid transport systems in plants is likely. In the halophyte *M. crystallinum*, expression of McNRT1 was stimulated by salt stress in both leaf and root tissue (Popova et al., 2003). Increased transcript abundance of this nitrate transporter in the root epidermis and the root vascular tissue indicates increased uptake and increased long-distance transport of nitrate in response to salt stress (Popova et al., 2003). In leaves, expression of McNRT1 increased in mesophyll cells and in the phloem (Popova et al., 2003). Compared with Arabidopsis, Thellungiella expresses the nitrate transporters NRT2.6 in the roots and a NAR2-like protein in the shoots at higher levels, and nitrate transporters were identified as a functional group with higher expression levels in Thellungiella than in Arabidopsis. An ammonium transporter (AMT1,3) also exhibits higher transcript abundance in Thellungiella than in Arabidopsis. Better N supply could help Thellungiella to maintain growth during salt stress and to produce more amino acids for osmotic balance of the cytoplasm.

Several amino acid, peptide and nucleotide transporters were also expressed at higher levels in Thellungiella plants than in Arabidopsis, e.g. ProT1, ENT3, ENT7, PTR7 and PUP10. Proline transporters (ProTs), are known to be induced by salt treatment in plants (Rentsch et al., 1996; Ueda et al., 2001). ProTs mediate the transport of the compatible solutes Pro, glycine betaine, and the stress-induced compound gamma-aminobutyric acid (Schwacke et al., 1999). All three AtProTs (AtProT1-3) are localized at the plasma membrane in Arabidopsis (Grallath et al., 2005). AtProT1 expression was found in the

phloem or phloem parenchyma cells throughout the whole plant, indicating a role in long-distance transport of compatible solutes. The ENT (equilibrative nucleoside transporter) family in Arabidopsis was studied by Wormit et al. (2004). AtENT4, AtENT6 and AtENT7 exhibit broad substrate specificity and transported the purine nucleosides adenosine and guanosine, as well as the pyrimidine nucleosides cytidine and uridine (Wormit et al., 2004). AtENT1 activity was clearly pH-dependent, AtENT3, 4 and 6 exhibited a less pronounced pH-dependency, and AtENT7 was not affected by changes in pH (Wormit et al., 2004). Higher expression of amino acid and nucleoside transporters in Thellungiella suggests that Thellungiella has enhanced capacity for the uptake and re-allocation of N-compounds, many of which are likely to act as compatible solutes. ANT1 (aromatic and neutral transporter) was characterised by (Chen et al., 2001). It transports not only aromatic and neutral amino acids and arginine, but also indole-3-acetic acid and 2,4-dichlorophenoxyacetic acid. Thus, amino acid transporters could link N homeostasis with phytohormone transport, thereby switching on the corresponding signalling pathway. In this context it is remarkable that auxin transporters also featured among transcripts with higher expression levels in Thellungiella than in Arabidopsis, both individually and as a group.

4.4.3.6 ABC transporters

ABC transporters constitute a large gene family of trans-membrane proteins containing a typical ATP-binding cassette signature. Five members of the MRP subfamily were constitutively expressed at higher levels in the roots of Thellungiella compared with Arabidopsis. They are homologues of AtMRP2, 3, 5, 6 and AtMRP14. MRP4 and MRP8 were expressed at higher levels in the shoots of Thellungiella than in Arabidopsis both with and without salt. MRPs is the second most highly represented subfamily of Arabidopsis full ABC transporters, with 15 members. This means that at least a third of

the MRP subfamily were expressed more abundantly in Thellungiella. Several members of the MRP subfamily have been cloned from Arabidopsis (Rea, 1999). AtMRP1, AtMRP2, and AtMRP3 encode Mg-ATP-energized pumps active in the transport of glutathione (GS) conjugates and other bulky amphipathic anions with various substrate selectivities and transport capacities (Lu et al., 1998; Lu et al., 1997; Tommasini et al., 1998). AtMRP2 localizes to the vacuolar membrane fraction from seedlings. The substrates of AtMRP2 include not only GS conjugates but also glucuronate conjugates demonstrated by heterologous expression in yeast (Liu et al., 2001). Conjugates with glutathione, glucuronide and glucose (in some cases) are final products of pesticides and other xenobiotics detoxification procedures in plants. They are secreted to the large central vacuole through ABC transporters, probably mainly MRPs (Tommasini et al., 1998). Lee et al. (2004) demonstrated AtMRP5 is a putative sulfonylurea receptor that is involved in K homeostasis and, thus, also participates in the NaCl stress response. When plant seedlings were treated with 100 mM NaCl, *atmrp5-2* seedlings accumulated less K and more Na than the wild type (Lee et al., 2004).

Apart from MRPs, there are also 3 MDRs (AtMDR6, AtMDR7 and AtMDR11) and 3 PDRs (AtPDR6, AtPDR8 and AtPDR10) that are expressed at higher levels in the roots of Thellungiella compared with Arabidopsis. MDRs are homologues to mammalian multidrug resistance proteins/ P-glycoproteins, many of which are plasma membrane efflux pumps functioning in the transport of amphipathic cations (Schinkel et al., 1997) and/or the translocation of cationic phospholipids between membrane bilayer leaflets (Ruetz and Gros, 1994; van Helvoort et al., 1996). More recently, members of the MDR/PGP subfamily of ABC transporters have been shown to function in the transport of the phytohormone auxin in plants (Geisler et al., 2005; Noh et al., 2001; Terasaka et al., 2005). PDRs are pleiotropic drug resistance proteins. An Arabidopsis PDR isoform,

AtPDR12 has been cloned and displayed distinct induction profiles after inoculation of plants with compatible and incompatible fungal pathogens and treatments with salicylic acid, ethylene, or methyl jasmonate (Campbell et al., 2003). Therefore ABC transporters could be the linking step between metabolism and various hormone mediated signalling pathways. As mentioned before many members of auxin transporter family were expressed more abundantly in both roots and shoots of Thellungiella than in Arabidopsis, suggesting that auxin signalling pathway could play an important role in Thellungiella growth and abiotic stress responses.

4.4.4 Similarity in the transcriptional response to salt stress between Arabidopsis and Thellungiella

Thellungiella is more tolerant to salt stress than Arabidopsis. When the two plants were treated with the same salt treatment, fewer genes were affected at the transcript level in Thellungiella compared with Arabidopsis. Or, in other words, salt-induced transcription changes of individual genes in Thellungiella were weaker or less significant than those in Arabidopsis, considering either fold-changes or FDR values. A general trend revealed by this study is that salt regulation of the membrane transporter transcriptomes in the two species is more similar in roots than in shoots, and that down-regulation of gene expression is more similar than up-regulation (Figure 4-5). Root cells are in direct contact with the soil environment. Under salt stress, the roots of Arabidopsis and Thellungiella were surrounded by the same solution, thus exposed to the same level of stress factor. This might explain why the salt response of root genes is similar in Arabidopsis and Thellungiella, both with respect to the strength of the response and with respect to the affected genes. Shoot cells experience salt stress indirectly, depending on the amount of salt taken up by the roots and transferred to the shoots. Both the rate of Na uptake into root cells and Na accumulation in the shoots are lower in

Thellungiella than in Arabidopsis (see Chapter 2). The shoot cells of Arabidopsis and Thellungiella therefore experience a different apoplastic environment under salt stress. This provides a good explanation for the observed difference in salt-induced expression changes of shoot genes between Thellungiella and Arabidopsis. From an energetic point of view, it is cheaper for a plant to switch off genes in order to cope with environmental stress than to produce new transcripts. This could be the reason for the fact that more genes were commonly depressed by salt stress than were commonly induced by salt in both species.

Nevertheless, the most strongly salt-responsive genes were fairly similar between the two species. Expression of 30% of the 100 most salt-responsive genes in Thellungiella roots were also regulated by salt (and in the same direction) in Arabidopsis roots. However, common regulation in the two species concerned mostly control genes. STP13, encoding a putative sugar transporter, was the only transporter gene commonly up-regulated by salt in the roots of both species. All other commonly up-regulated genes in either roots and shoots were typical abiotic stress induced genes, such as PIN2, P5CS and COR78 which were included in the probe set as controls. Although only a small number of known stress-induced genes were represented on the AMT array, it seems that typical stress-induced genes are more sensitive to moderate stress levels, are reduced earlier and/or are regulated more strongly than membrane transporter genes. More membrane transporters were among the genes that were commonly down-regulated by salt in the two species. In the roots aquaporins were down-regulated in response to salt stress in both Arabidopsis and Thellungiella, probably to minimize water loss. Salt-induced down-regulation of amino acid transporters, ABC transporter and peptide transporter in the shoots of both species probably functions in osmoadaptation, metabolic responses or signal transduction. In summary, the salt-

induced basic stress response concerning osmoregulation and metabolism are common between Arabidopsis and Thellungiella, whereas the active mechanisms in response to salt stress concerning the induction of specific functions in particular those related to ion homeostasis appear to be different between the two species.

4.4.5 Difference in the transcriptional response to salt stress between Arabidopsis and Thellungiella

Figure 4-6 summarizes salt-induced transcript regulation of ion transporters that was specific for Arabidopsis or Thellungiella. Species-specific salt induced changes in transcripts of ion transporters occurred mostly in the shoots of Arabidopsis. KUP/HAK/KT6, CAX3, NAR2-like protein and two putative sulphate transporters were up-regulated indicating Arabidopsis re-allocates inorganic nutrients during salt stress, such as K, Ca, N and S. Meanwhile the observed down-regulation of AHA2, CNGC8, CHX15 and a Cl channel might reflect inhibition of the uptake of toxic Na and Cl ions into shoot cells. Except for one metal transporter, AtMTPA1, which was down-regulated, no ion transporters was regulated by salt stress in the roots of Arabidopsis. Therefore, no effective regulation of ion transport seems to operate at the transcriptional level in Arabidopsis roots under salt stress. No evidence for an induction of Na export by salt stress was found in Arabidopsis. Na efflux systems, such as SOS1 are considered essential for salt resistance in Arabidopsis. The Arabidopsis knock-out mutant *sos1* is overly sensitive to salt stress (Shi et al., 2000), and over-expression of Na-H antiporters improves the salt tolerance of plants (Shi et al., 2000; Apse et al., 1999). The regulatory mechanism for SOS1 involves a Ca binding protein (SOS3) and a Ca-dependent protein kinase (SOS2) (Qiu et al., 2002). Whether SOS1 is also regulated at the transcript level is less clear (Maathuis et al., 2003). As pointed out by Munns at al. (2006), Na efflux has to function even in low salt conditions in order to keep the majority of Na ions away

from the cytosol. Enhancing the electrochemical gradient of protons across the plasma membrane, e.g. through up-regulation of AHA2, could effectively increase Na efflux through Na-H antiporters, so that enhanced expression of Na-H antiporters is not necessary.

In contrast to Arabidopsis, transcriptional regulation of ion transport in response to salt stress by Thellungiella occurs mostly in the roots. After application of 100 mM NaCl, CNGC5, CNGC8 and a putative metal transporter ZIP8 were down-regulated, suggesting that these genes might encode pathways for Na uptake into root cells of Thellungiella. The tissue specific regulation of CNGC8 in Arabidopsis (shoots) and Thellungiella (roots) is particularly interesting. Assuming that CNGC8 provides a pathway for Na uptake it appears that Thellungiella protects its cells from toxic Na uptake through this transporter in roots whereas Arabidopsis takes this measure only in the leaves.

In conclusion, both Arabidopsis and Thellungiella try to prevent Na influx during salt stress. Arabidopsis cannot prevent Na entering the root cells, therefore apoplastic Na concentrations in the surrounding of shoot cells increase quickly after salt stress triggering transcriptional responses of shoot cells. Thellungiella can successfully prevent Na from entering root cells, so that shoot cells are not subject to salt stress as quickly as in Arabidopsis, and respond less at the transcriptional level. Therefore Thellungiella shoots have enough time to grow in order to dilute the vacuolar Na concentration. CNGC8 emerges as an interesting candidate for a Na influx pathway. It should be cloned from both Arabidopsis and Thellungiella and studied in detail with respect to its role and regulation during salt stress.

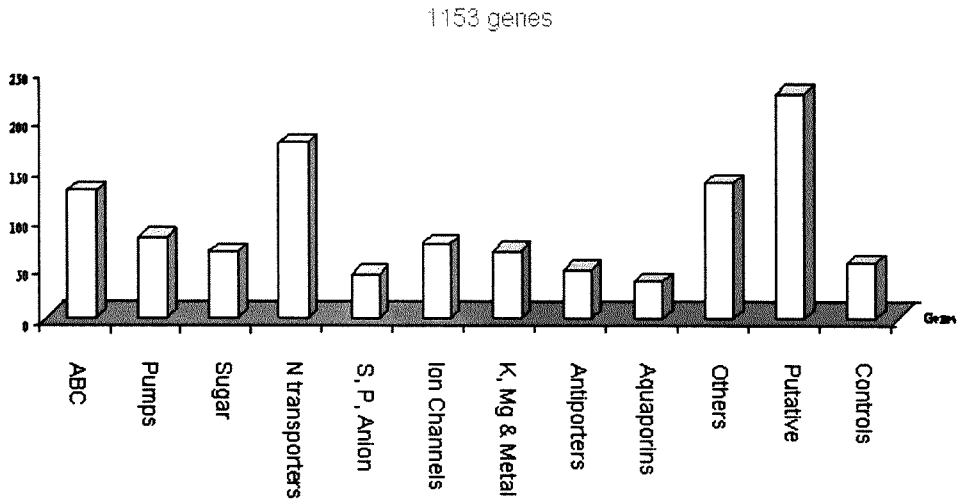

Figure 4-1. Numbers of genes in different transporter families represented by the AMT array.

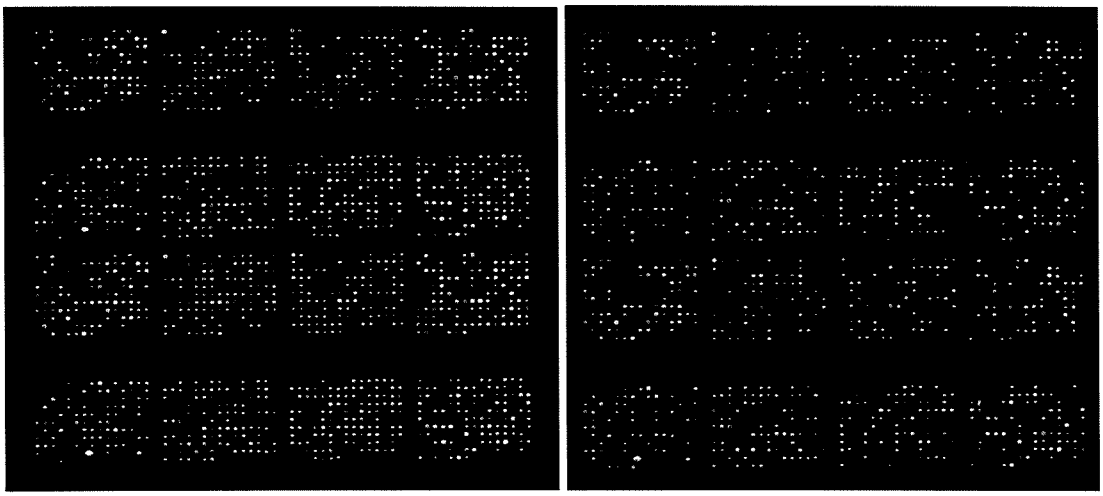

Figure 4-2. Overlay images of AMT arrays hybridised with Cy3 (control, green) and Cy5 (+NaCl, red) labeled root cDNA samples from *A. thaliana* (left) and *T. halophila* (right).

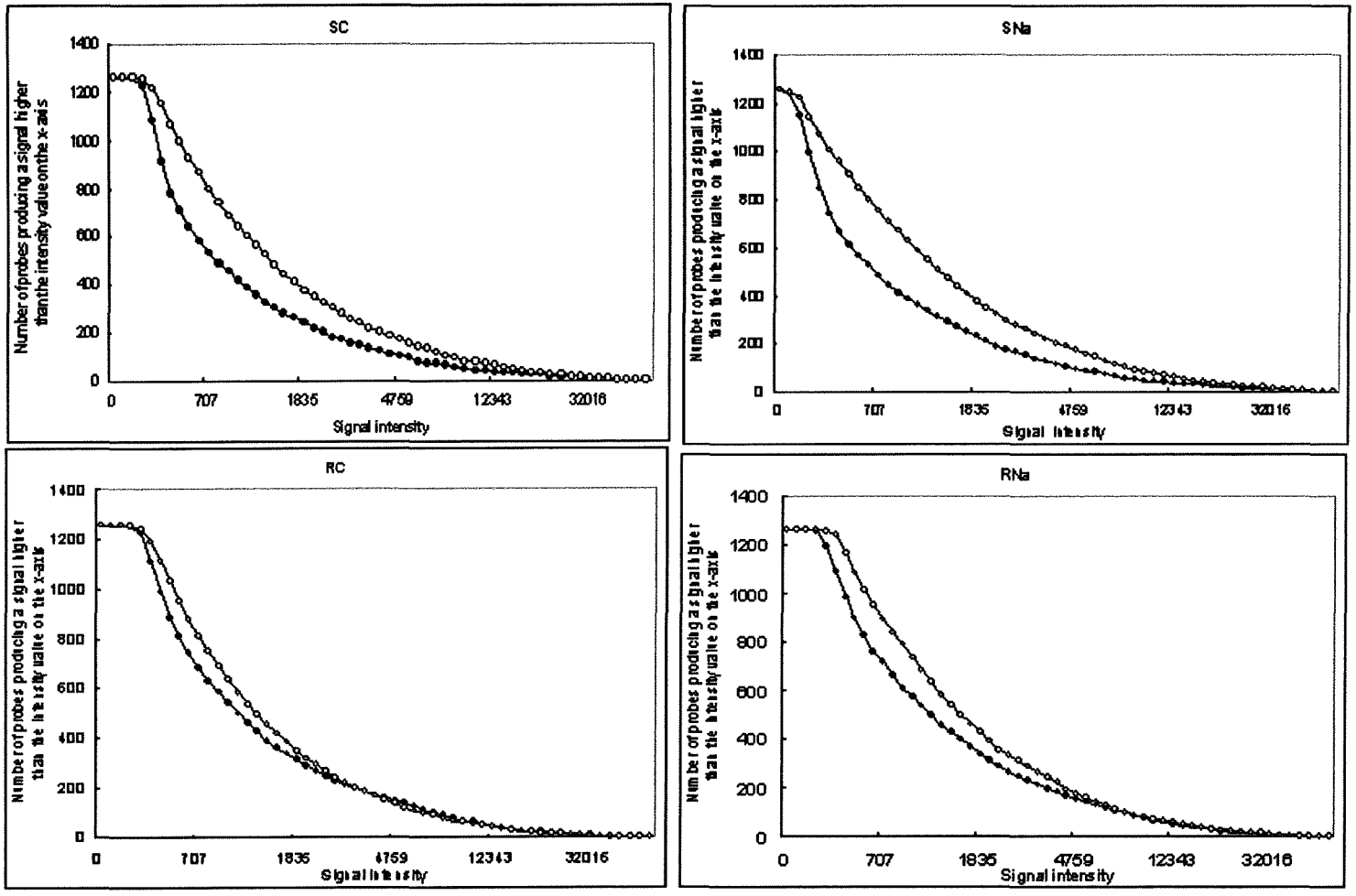

Figure 4-3. Distribution of hybridization signals. The numbers of genes above a certain signal intensity were plotted against signal intensity to compare the distributions of hybridisation signal derived from AMT arrays hybridized with cDNA from Arabidopsis or Thellungiella grown with or without salt. Arabidopsis: open-circle. Thellungiella: closed-circle. SC: shoot control. SNa: shoot salt-treated. RC: root control. RNa: root salt-treated.

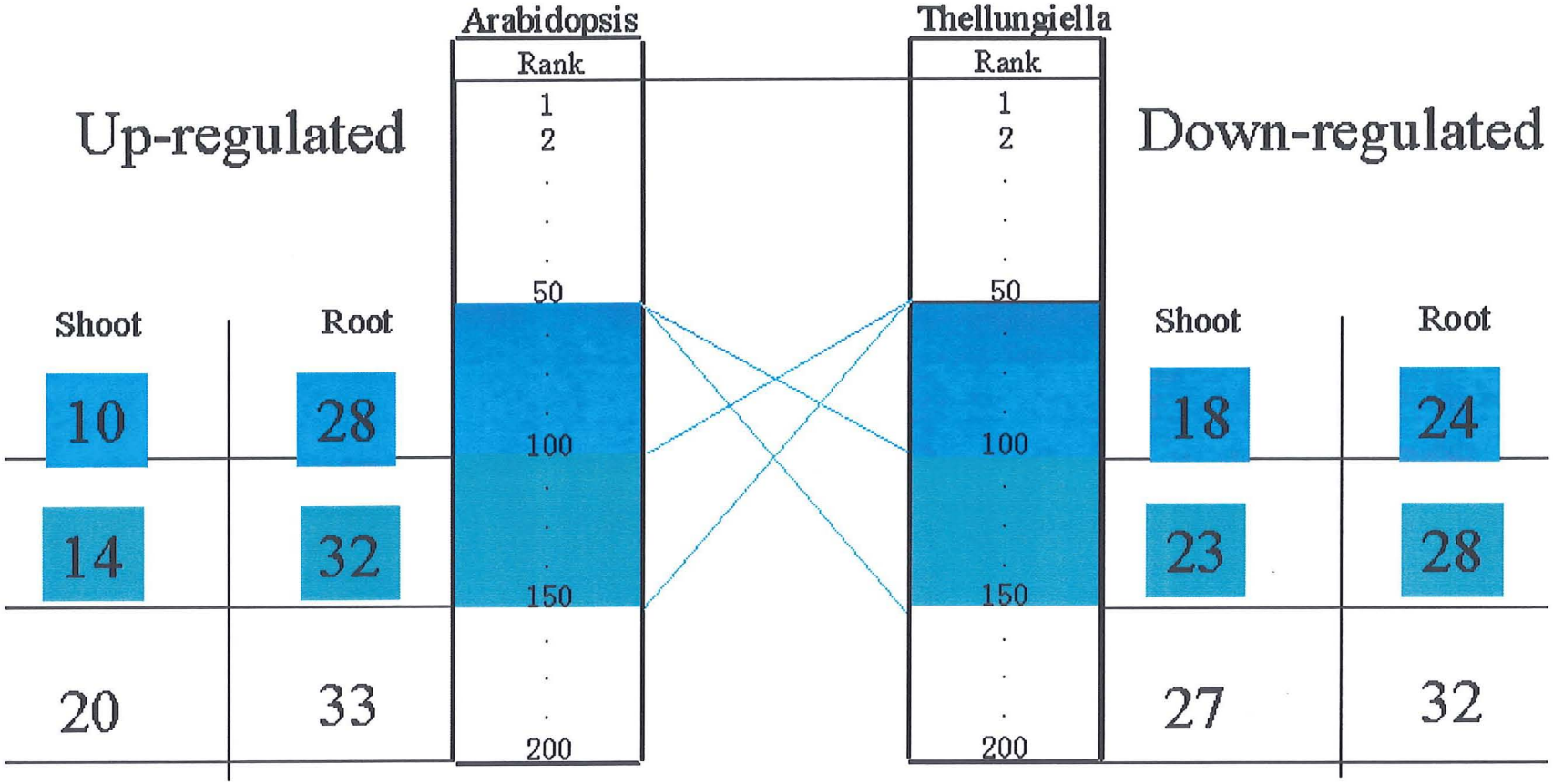

Figure 4-4.Numbers of commonly regulated transporter genes in Arabidopsis and Thellungiella. The numbers of genes are shown that were positioned within the top 50 genes of the RP ranked list for one species and within the top 100, 150 and 200 genes for the other species.

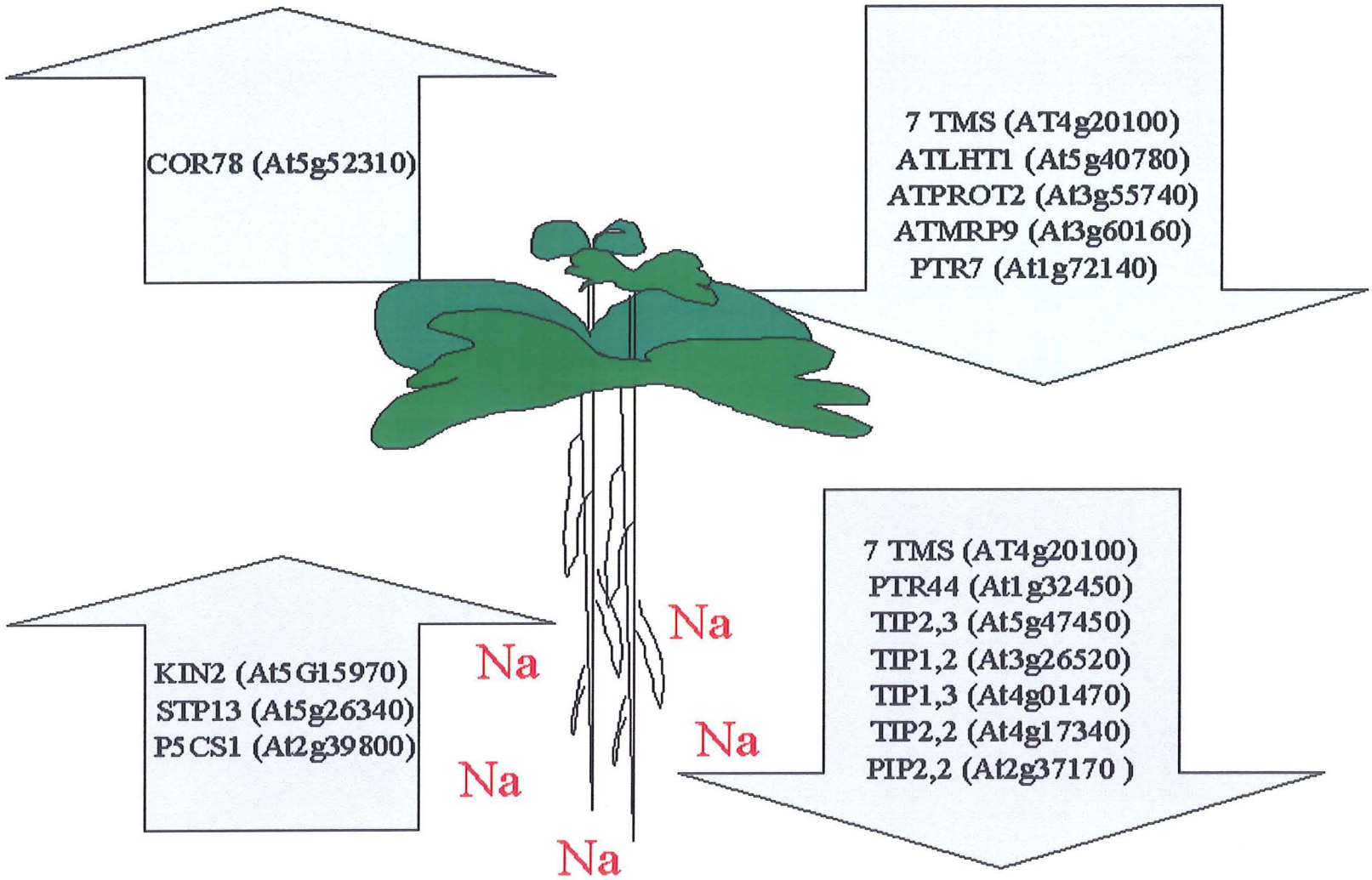

Figure 4-5. Transcripts that were changed in both Arabidopsis and Thellungiella after a 24 h treatment with 100 mM NaCl (FDR<30%).

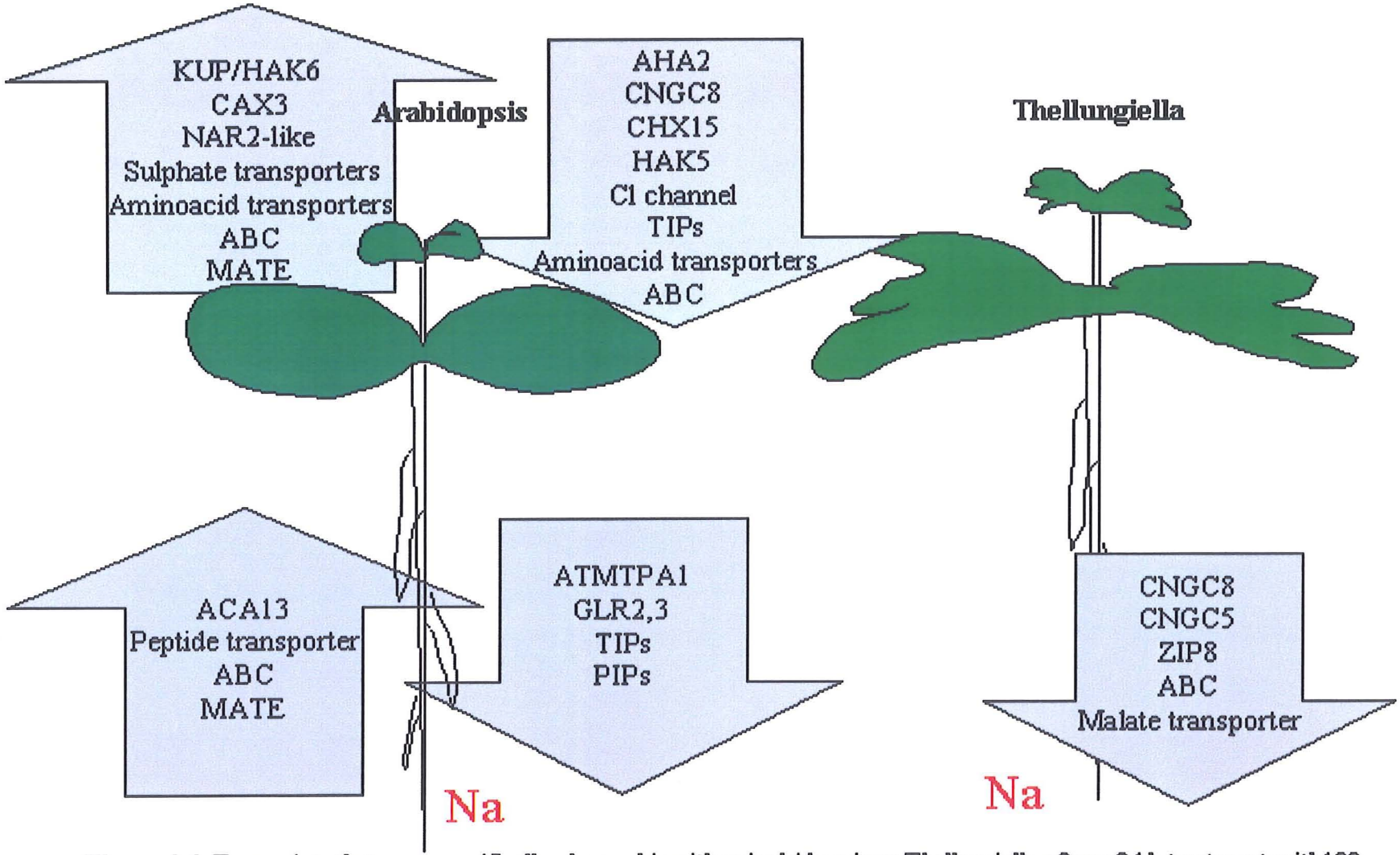

Figure 4-6. Transcripts that were specifically changed in either Arabidopsis or Thellungiella after a 24 h treatment with100 mM NaCl (FDR<10%).

Table 4-1. Sample hybridization for the miroarray experiments. The hybridized arrays were named after the tissue and species of the RNA samples. Green background indicates label with Cy3 (control channel). Red background indicates label with Cy5 (treated channel).

	A. thal.		*T. halo.*	
	Shoot	Root	Shoot	Root
Control	MNS	MNS	MNS	MNS
Treatment	+ 100 mM NaCl	+ 100 mM NaCl	+ 100 mM NaCl	+ 100 mM NaCl
Name	AtS	AtR	ThS	ThR

Table 4-2. The mastermix used to label cDNA with Cy-dyes during reverse transcription.

	µl/reaction
5x reverse transcription buffer	8
dNTP mix (5 µl of 100 mM dA-, dG-, dTTP, 2 µl 100 mM dCTP and 83 µl Rnase-free water)	4
Cy3 or Cy5 labelled dCTP	2
0.1 M DTT	4
Total	18

Table 4-3. Buffers used for washing the AMT array after hybridization.

	Components
Washing buffer 1	2 × SSC, 0.1% SDS
Washing buffer 2	1 × SSC
Washing buffer 3	0.5 × SSC

Table 4-4. Transporter genes with higher transcript abundance in Thellungiella than in Arabidopsis. '+' means that the gene has been identified as differentially expressed when comparing control and/or +NaCl plants. E-values and FDR (false discovery rates) relate to the comparison of control plants if the gene showed a significant difference between Thellungiella and Arabidopsis, otherwise they refer to +NaCl treated plants. The genes with FDR<1% in the roots and FDR<10% in the shoots are presented.

	AGI	Description	Control	+NaCl	E-value	FDR(%)
Root	At3g01390	V-type pump (VHA-G1)	+	+	0	0
	At2g16510	V-type pump (VHA-c5)	+	+	0	0
	At4g23710	V-type pump (VHA-G2)	+	+	0	0
	At4g38920	V-type pump (VHA-c3)	+	+	0.012	0.1
	At3g32990	V-type pump (c2)	+	+	0.02	0.1
	At1g78900	V-type pump (VHA-A)	+	+	0.024	0.1
	At4g11150	V-type pump (VHA-E1)	+	+	0.04	0.2
	At5g47030	V-type pump (delta c)	+	+	0.048	0.2
	At4g34720	V-type pump (VHA-c1)	+	+	0.068	0.3
	At1g12840	V-type pump (VHA-C)	+	+	0.096	0.3
	At1g75630	V-type pump (VHA-c4)	+	+	0.244	0.7
	At5g13450	V-type pump (delta M)	+	+	0.268	0.7
	At2g25610	V-type pump (VHA-c"2)	+	+	0.32	0.8
	At1g15690	Ppase (AVP1)	+	+	0.024	0.1
	At2g23280	P-type pump	+	+	0	0
	At1g59820	P-type pump(ALA3)	+	+	0.024	0.1
	At3g13080	ABC(ATMRP3)	+	+	0	0
	At2g34660	ABC(ATMRP2)	+	+	0	0
	At1g04120	ABC(ATMRP5)	+	+	0.004	0
	At3g30842	ABC(ATPDR10)	+	+	0.004	0
	At3g21250	ABC(ATMRP6)	+		0.004	0
	At3g28860	ABC(ATMDR11)	+	+	0.044	0.2
	At2g36380	ABC(ATPDR6)	+	+	0.048	0.2
	At3g59140	ABC(ATMRP14)	+	+	0.068	0.3
	At5g46540	ABC(ATMDR7)	+	+	0.212	0.6
	At1g59870	ABC(ATPDR8)	+	+	0.408	0.9
	At2g07680	ABC(ATMRP11)		+	0.2	0.5
	At1g15520	ABC(ATPDR12)		+	0.264	0.7
	At1g30840	Aminoacid transporter (PUP10)	+	+	0	0
	At1g61630	Aminoacid transporter (ATENT7)	+	+	0	0
	At3g28960	Aminoacid transporter	+	+	0.004	0
	At3g24300	Ammonium transporter (AMT1,3)	+		0.108	0.3

Table 4-4 continues on next page

Table 4-4 continued.

	AGI	Description	Control	+NaCl	E-value	FDR(%)
	At2g39890	Aminoacid transporter (ATPROT1)	+		0.348	0.8
	At2g13650	Sugar transporter(SUGAR5)	+	+	0	0
	At5g61520	Sugar transporter(STP3)	+	+	0.104	0.4
	At2g20780	Sugar transporter(STP38)	+	+	0.128	0.4
	At3g19930	Sugar transporter(STP4)		+	0.04	0.2
	At2g40460	Peptide transporter(PTR17)		+	0.412	0.9
	At1g77690	Auxin transporter (ATAUXR3)	+	+	0.048	0.2
	At2g21050	Auxin transporter (ATAUXR2)	+		0.4	0.9
	At2g32830	Phosphate transporter (PHT1.5)	+	+	0.004	0
	AT5g14570	Nitrate tansporter(NRT2.6)	+	+	0.004	0
	At1g15460	Putative anion exchanger	+	+	0.248	0.7
	At3g46900	Metal transporter(COPT3)	+	+	0.104	0.3
	At4g19690	Metal transporter(IRT1)	+	+	0.192	0.6
	At2g46800	Metal transporter(ATMTP1)	+	+	0.232	0.6
	At3g04800	Inner mitoch membrane protein family	+		0.232	0.7
Shoot	At5g14880	KUP/HAK/KT8	+	+	0	0
	At3g13090	ABC(ATMRP8)	+	+	0.008	0.2
	At2g47800	ABC(ATMRP4)	+	+	0.04	0.5
	At2g01320	ABC(ATWBC7)		+	1.736	9.6
	At2g16850	PIP2,8	+	+	0.004	0.1
	At5g60660	PIP2,4	+		0.5	3.8
	At3g32990	V-type pump (c2)	+	+	0.012	0.2
	At4g30190	P-type pump(AHA2)	+	+	0.688	4.3
	At4g05110	Aminoacid transporter(ATENT3)	+	+	0.016	0.3
	At1g48370	Aminoacid or metal transporter	+	+	0.072	0.7
	At1g16390	Sugar transporter	+	+	0.028	0.4
	At2g28180	Cation-H antiporter(CHX8)	+	+	0.072	0.8
	At5g55470	Na-H antiporter(NHX3)	+	+	0.544	3.9
	At1g80900	Mg-transporter(MGT1)	+		0.32	2.9
	At5g50200	Nitrate tansporter (NAR2-LIKE 1)	+		0.86	4.8
	At1g23090	Sulphate transporter (SULTR3.3=AST91)	+		1.588	8.4
	At3g06460	8 TMS putative	+	+	0.496	4.1
	At1g03550	Secretory carrier family		+	1.036	6.1

Table 4-5. Transporter groups identified by iGA to give higher signal intensities in Thellungiella than Arabidopsis. 'Number' shows total number of genes in the group. 'Changed' shows the number of genes that gave higher signal intensities in Thellungiella. '+' means that the gene has been identified as differentially expressed when comparing control and/or +NaCl plants. P-values and numbers relate to the comparison of control plants if the gene showed a significant difference between Thellungiella and Arabidopsis, otherwise they refer to +NaCl treated plants. (P<0.015).

	Group Name	Number	Changed	p-value	Control	+NaCl
Root	V-type pump	32	10	2.82E-08	+	+
	Auxin transporter	12	7	0.007164	+	+
	MATE family	55	55	0.007176	+	
	Nitrate tansporter	9	3	0.013019	+	+
	PPase	3	3	0.014595	+	+
	Sugar transporter	67	12	0.007901		+
	Metal transporter	38	7	0.012838		+
Shoot	Nitrate tansporter	9	3	0.000602	+	+
	aquaporin	38	6	0.001997	+	+
	Auxin transporter	12	4	0.009044	+	+
	Secretory carrier family	4	2	0.010388	+	+
	K-transporter	13	1	0.011706	+	+
	Glutamate receptor	20	20	0.013203	+	+

Table 4-6. Transporter genes with lower transcript abundance in Thellungiella than in Arabidopsis. '–' means that the gene has been identified as differentially expressed when comparing control and/or +NaCl plants. E-values and FDR (false discovery rates) relate to the comparison of control plants if the gene showed a significant difference between Thellungiella and Arabidopsis, otherwise refer to +NaCl treated plants. The genes with FDR<1% in the roots and FDR<10% in the shoots are presented.

	AGI	Description	Control	+NaCl	E-value	FDR(%)
Root	At3g22910	P-type pump(ACA13)	–	–	0	0
	At2g22950/ At2g22960	P-type pump(ACA7)	–	–	0	0
	At3g63380	P-type pump(ACA12)	–	–	0	0
	At3g21180	P-type pump(ACA9)	–	–	0	0
	At2g41560	P-type pump(ACA4)	–	–	0	0
	At4g29900	P-type pump(ACA10)	–	–	0	0
	At5g57110	P-type pump(ACA8)	–	–	0.004	0
	At3g57330	P-type pump(ACA11)	–	–	0.004	0
	At1g27770	P-type pump(ACA1/PEA1)	–	–	0.044	0.2
	At1g07670&At g07810	P-type pump (ECA4&ECA1/ACA3)	–		0.168	0.5
	At4g00901	P-type pump (ECA2/ACA6)	–	–	0.176	0.5
	At4g37640	P-type pump(ACA2)	–	–	0.248	0.6
	At1g63440	P-type pump(HMA5)	–	–	0.016	0.1
	At4g30110	P-type pump(HMA2)	–	–	0.016	0.1
	At4g33520	P-type pump(PAA1)	–	–	0.096	0.3
	At5g13580	ABC(ATWBC6)	–	–	0	0
	At2g37010	ABC(NAP12)	–	–	0	0
	At4g30300	ABC(NAP15)	–	–	0	0
	At2g13610	ABC(ATWBC5)	–	–	0	0
	At1g65410	ABC(NAP11)	–	–	0	0
	At5g14100	ABC(NAP14)	–	–	0	0
	At1g63270	ABC(NAP10)	–	–	0.004	0
	At4g25750	ABC(ATWBC4)	–	–	0.024	0.1
	At4g25450	ABC(NAP8)	–	–	0.024	0.1
	At5g18290	SIP1,2	–	–	0.004	0
	At5g37810	NIP4,1	–	–	0.004	0
	At4g19030	NIP1,1	–	–	0.004	0
	At3g06100	NIP7,1	–	–	0.024	0.1
	At2g21020	NLM9	–	–	0.04	0.2
	At1g73190	TIP3,1	–	–	0.048	0.2
	At1g52180	TIP	–	–	0.064	0.2
	At2g29870	NLM3	–	–	0.076	0.3
	At1g80760	NIP6,1	–	–	0.08	0.3
	At1g31880	NIP3,1	–		0.276	0.7
	At3g19640	Mg-transporter(MGT4)	–	–	0.224	0.6

Table 4-6 continues on next page

Table 4-6 continued.

	AGI	Description	Control	+NaCl	E-value	FDR(%)
	At5g09710	Mg-transporter(MGT7)	–	–	0.224	0.6
	At5g22830	Mg-transporter(MGT10)	–	–	0.232	0.6
	At5g64560	Mg-transporter(MTG9)		–	0.284	0.7
	At5g09690	Mg-transporter(MTG8)		–	0.292	0.7
	At1g57550	Stress induced	–	–	0.068	0.2
	At2g24040	Stress induced	–	–	0.276	0.7
	At5g01680	Cation-H antiporter(CHX26)	–	–	0.332	0.8
	At1g05580	Cation-H antiporter(CHX23)		–	0.344	0.8
	At2g13620	Cation-H antiporter(CHX15)		–	0.412	0.8
	At3g53720	Cation-H antiporter(CHX21)		–	0.432	0.9
	At1g14660	Na-H antiporter(NHX8)		–	0.368	0.8
	At2g04070	In MATE family		–	0	0
	At4g37030	6 TMS putative		–	0.184	0.5
Shoot	At5g52310	COR78	–	–	0	0
	At5g15970	KIN2	–	–	0	0
	At3g05880	LTI6A	–	–	0.02	0.5
	At1g01620	PIP1,3	–	–	0.04	0.8
	At2g36830	TIP1,1	–	–	0.128	1.8
	At3g53420	PIP2,1	–	–	0.684	6.8
	At2g39010	PIP2,6	–	–	0.912	6.5
	At1g51500	ABC(ATWBC12)	–	–	0.068	1.1
	At1g59870	ABC(ATPDR8)	–	–	0	0
	At3g47730	ABC(ATATH1)		–	1.228	5.6
	At4g09810	In NST-TPT family	–	–	0.544	6
	At1g76670	In NST-TPT family	–	–	1.42	7.9
	At3g07390	6 TMS putative	–	–	0.152	1.9
	At4g12980	6 TMS putative	–		0.7	5.8
	At4g04340	10 TMS putative	–	–	0.912	7
	At3g54510	10 TMS putative	–	–	1.04	6.9
	At1g30360	10 TMS putative	–	–	1.692	8.5
	At4g39460	MC family	–		1.12	7
	At2g20780	Sugar transporter(STP38)	–	–	1.356	8
	At4g35300	Sugar transporter(SUPGAR2)		–	1.924	8.4
	At3g21670	Peptide transporter PTR36	–	–	1.636	8.6
	At1g69870	Peptide transporter(PTR15)		–	0.48	4.8
	At4g38250	Aminoacid transporter	–		2.076	9.9
	At5g47030	V-type pump (delta c)	–	–	0.684	6.2
	At5g65380	In MATE family		–	0.148	2.5
	At1g12950	In MATE family		–	1.212	5.8
	At2g37410	In MPT family		–	0.688	4.9

Table 4-7. Transporter groups identified by iGA with lower signal intensities in Thellungiella than in Arabidopsis. 'Number' shows total number of genes in the group. 'Changed' shows the number of genes that showed lower signal intensities in Thellungiella. (P<0.015).

	Group Name	Number	Changed	p-value
Root	P-type pump	48	10	3.22E-08
	Cation-H antiporter	28	13	5.87E-08
	aquaporin	38	12	1.35E-07
	Mg-transporter	11	7	2.07E-06
	Na-H antiporter	10	6	3.64E-05
	Ca-H antiporter	11	5	0.000882
	K-efflux system	6	5	0.003559
	Putative Ca channel	8	7	0.003789
	Sulphate transporter	14	10	0.008249
	Glutamate receptor	20	19	0.009047
	CNGC	20	14	0.009523
	K-transporter	13	8	0.011901
Shoot	aquaporin	38	4	0.000475
	Stress induced	6	3	0.001144
	Ammonium transporter	6	5	0.004073
	In NST-TPT family	12	2	0.011816
	CNGC	20	18	0.015599

Table 4-8. Numbers of transporter genes that showed similar regulation by salt in both Arabidopsis and Thellungiella with FDRs smaller than 10% in one species and smaller than 10%, 20% or 30% in the other species.

FDR	Root		Shoot	
	Up	Down	Up	Down
< 10%	3	3	0	3
< 20%	3	5	1	3
< 30%	3	7	1	5

Table 4-9. Transporter genes up-regulated in the shoots of Arabidopsis by treatment with100 mM NaCl for 24h (FDR≤10%). No gene was induced in the shoots of Thellungiella (FDR≤10%).

AGI	Description	E-value	FDR(%)
At5g52310	COR78*	0.408	3.7
At2g39800	P5CS1	0.452	3.5
At1g70300	KUP/HAK/KT6	0.02	0.5
At3g51860	Ca-H antiporter (CAX3)	0.924	5.4
At5g50200	Nitrate tansporter (NAR2-LIKE1)	0.32	3.6
At3g51900	Sulphate transporter (SULTR3.1/AST12)	0.348	3.5
At5g47560	Other anion transporter (NA-SULFATE)	0.528	3.8
At2g41190	Aminoacid transporter	0	0
At5g65990	Aminoacid transporter	0.724	4.8
At1g58360	Aminoacid transporter (ATAAP1)	0.736	4.6
At1g69870	Peptide transporter (PTR15)	0.22	3.7
At1g71960	ABC (ATWBC26)	1.728	8.6
At3g16340	ABC (ATPDR1)	1.832	8.7
At5g65380	In MATE family	0.204	4.1
At4g39030	In MATE family	2.296	10
At3g20300	6 TMS putative	0	0
At1g78610	7 TMS putative	0.232	3.3
At4g21570	7 TMS putative	0.236	3

* these genes were found commonly regulated by salt treatment in both species in the same direction with FDR < 30%.

Table 4-10. Transporter genes up-regulated in the roots of Thellungiella and Arabidopsis by treatment with100 mM NaCl for 24 h (FDR<10%).

	AGI	Description	E-value	FDR(%)
T. halophila	At5G15970	KIN2*	0	0
	At1G77120	ADH1	0.124	3.1
	At2g39800	P5CS1*	0.568	9.5
	At5g26340	Sugar transporter (STP13)*	0.116	3.9
	At1g12730	7 TMS putative	0.312	6.2
A.thaliana	At5g15970	KIN2*	0	0
	At2g39800	P5CS1*	0.156	2.2
	At3g22910	P-type pump(ACA13)	1.384	8.1
	AT5g26340	Sugar transporter(STP13)*	0.012	0.2
	At1g59740	Peptide transporter (PTR35)	0.876	5.8
	At3g13080	ABC(ATMRP3)	0	0
	At1g71330	ABC(NAP5)	0	0
	At1g02520	ABC(ATMDR8)	0.228	2.3
	At2g47000	ABC(ATMDR4)	0.324	2.9
	At3g62150	ABC(ATMDR17)	1.14	7.1
	At2g04070	In MATE family	0	0
	At1g12950	In MATE family	0.592	4.2
	AT4g37030	6 TMS putative	0.132	2.2
	AT5g35735	6 TMS putative	0.212	2.7

* these genes were found commonly regulated by salt treatment in both species in the same direction with FDR < 30%.

Table 4-11. Transporter genes down-regulated in the shoots of Thellungiella and Arabidopsis by treatment with100 mM NaCl for 24 h (FDR<10%).

	AGI	Description	E-value	FDR(%)
T. halophila	At4g20100	7 TMS putative*	0	0.0
	At3g13090	ABC (ATMRP8)	0.052	2.6
	At3g55740	Aminoacid transporter (ATPROT2)*	0.204	5.1
	At5g40780	Aminoacid transporter (ATLHT1)*	0.244	4.9
	At2g39130	Aminoacid transporter	0.336	5.6
A. thaliana	At3g16240	Aquaporin (TIP2,1)	0.016	0.3
	At4g01470	Aquaporin (TIP1,3)	0.04	0.7
	At2g25810	Aquaporin (TIP4,1)	0.076	1.1
	At3g26520	Aquaporin (TIP1,2)	0.112	1.4
	At4g35100	Aquaporin (PIP2,7)	1.616	7.3
	At4g13420	K-transporter (HAK5)	1.592	7.6
	At1g19780	CNGC8	0	0
	At2g13620	Cation-H antiporter (CHX15)	0.004	0.1
	At4g30190	P-type pump (AHA2)	0.116	1.3
	At5g57490	Cl channel (PORIN3)	1.108	6.5
	At1g72140	Peptide transporter (PTR7)*	0	0
	At1g52190	Peptide transporter (PTR20)	0.216	2
	At5g40780	Aminoacid transporter (ATLHT1)*	0.208	2.1
	At3g55740	Aminoacid transporter (ATPROT2)*	0.272	2.1
	At1g08230	Aminoacid transporter (ATAAP10)	1.184	6.2
	At5g49630	Aminoacid transporter (ATAAP6)	1.832	8
	At3g28860	ABC (ATMDR11)	0.232	1.9
	At3g28360	ABC (ATMDR18)	0.396	2.8
	At4g20100	7 TMS putative*	0	0
	At4g12980	6 TMS putative	0.596	4

* these genes were found commonly regulated by salt treatment in both species in the same direction with FDR < 30%.

Table 4-12. Transporter genes down-regulated in the roots of Thellungiella and Arabidopsis by treatment with100 mM NaCl for 24 h in (FDR<10%).

	AGI	Description	E-value	FDR(%)
T. halophila	At3g16240	Aquaporin (TIP2,1)	0.016	0.4
	At5g47450	Aquaporin (TIP2,3)*	0.188	3.1
	At1g19780	CNGC8	0.064	1.3
	At5g57940	CNGC5	0.94	10.4
	At1g31260	Metal transporter (ZIP8)	0.62	7.8
	At5g64280	Malate transporter	0	0
	At1g32450	Peptide transporter (PTR44)*	0	0
	At1g72140	Peptide transporter (PTR7)	0.612	8.7
	At3g28860	ABC (ATMDR11)	0.984	9.8
	At1g15210	ABC (ATMDR7)	1.048	9.5
	At4g20100	7 TMS putative	0	0
A. thaliana	At5g47450	Aquaporin(TIP2,3)*	0	0
	At4g17340	Aquaporin(TIP2,2)*	0.012	0.6
	At4g01470	Aquaporin(TIP1,3)*	0.36	4.5
	At2g37170	Aquaporin(PIP2,2)*	0.064	1.6
	At2g37180	Aquaporin(PIP2,3)	0.22	3.7
	At5g60660	Aquaporin(PIP2,4)	0.364	4
	At4g19030	Aquaporin(NIP1,1)	0.08	1.6
	At3g58810	Metal transporter(ATMTPA1)	1.168	9.7
	At1g32450	Peptide transporter(PTR44)*	0.292	4.2
	AT5g09220	Aminoacid transporter(ATAAP2)	0.94	8.5
	At2g24710	Glutamate receptor(GLR2.3)	0.428	4.3
	AT4g20100	7 TMS putative*	0.012	0.4

* these genes were found commonly regulated by salt treatment in both species in the same direction with FDR < 30%.

Table 4-13. Transporter families changed as groups in Arabidopsis and Thellungiella in response to 24 h treatment with100 mM NaCl as identified by iGA (P<0.015).

		A. thaliana	*T. halophila*
Root	Down	aquaporin V-type pump Sulphate transporter Cl channel MPT family	aquaporin Cl channel P-type pump Aminoacid or metal transporter Other anion transporter CNGC Condition, Sugar, Invertase K channel V-type pump
	Up	MATE family FBT family Phosphate transporter	MATE family
Shoot	Down	aquaporin Aminoacid transporter Putative anion exchanger	Aminoacid transporter
	Up	Sulphate transporter FBT family Ca-H antiporter 10 TMS MFS family	Secretory carrier family Stress induced Inner mitoch membrane protein family Auxin transporter

Chapter 5 Conclusions and Outlook

Salt stress is one of the most threatening environmental stresses reducing the global food production. Understanding mechanisms of salt tolerance in halophytic plants is a requirement for developing crop species with increased salt tolerance. This study focused on investigating ion transport features in a halophytic relative of Arabidopsis, both at physiological and transcriptional level.

I first identified Na uptake as a crucial parameter differing between the glycophyte and halophyte. After analysing components of Na transport with respect to their individual kinetic and pharmocological properties, I employed a microarray approach to identify targets for future molecular identification of ion transporters that have crucial function in salt stress adaptation.

A comparative approach was adopted in this study using the glycophytic model plant *Arabidopsis thaliana*, and its halophytic close relative, *Thellungiella halophila*. Net ion uptake and unidirectional Na fluxes during salt stress were analyzed in the two species using hydroponically cultured plants. Furthermore, transcriptional profiles of ion transporters under control and high-salt conditions were compared between the two species.

Such a comparative approach has obvious advantages over studies using only one species, e.g. Arabidopsis, barley or Mesembryanthemum. Previously I compared salt-sensitive with salt-tolerant cultured Arabidopsis cell lines (MRes project), but the relevance of this approach is questionable as any salt tolerance mechanisms in this system are limited to the cellular level and to the adaptive potential of a glycophyte. For example, callus developed from salt-tolerant cultured cells was found to be still sensitive to salt (Chandler and Thorpe, 1986).

The advantages of the Arabidopsis/Thellungiella system are:

1. One of the two species, *Arabidopsis thaliana*, is the best studied model plant. Its entire genome has been sequenced and is well annotated. A huge amount of information on the physiology and molecular biology of Arabidopsis is available especially with respect to ion transport and its regulation. Many important genes have been cloned and characterised, including ion transporters. Many tools and resources are available for comprehensive studies, e.g. molecular biology tools and transformation protocols, compilations of gene expression patterns, protein signatures and biochemical pathways.
2. The second species, *Thellungiella halophila*, is a true extremophile. It tolerates severe environmental stresses such as cold, drought and salinity. Specialized organs (e.g. salt glands) or modified carbon fixation (e.g. CAM) are *not* required for salt tolerance for Thellungiella.
3. The two species are very similar, both at the morphological and at the molecular level. This made comparative studies valid, and will facilitate further molecular studies.

The growth conditions were optimised so that Arabidopsis and Thellungiella plants developed at a similar speed. Growing plants in hydroponic culture allowed precise control of the ionic medium surrounding the roots. The hydroponic growth condition is more physiological than other *in vitro* systems i.e. petri dish agar plates, as it allows plants to transpire and to progress through to all different developmental stages.

One of the biggest challenges of any comparative study is the appropriate statistical treatment of the obtained data. Therefore I made a special effort to apply a number of quantitative procedures to the obtained raw data. The analyses included:

- Pairwise comparison of replicated parameters between the species: pairwise t-test.
- Multifactorial comparison: 3-way ANOVA.
- Kinetic analysis: curve fitting.
- Microarray analysis: quantile normalization, RP and iGA.
- Quantitive comparison of net and unidirectional Na uptake.

These vigorous analysis procedures reliably filtered the large data set for meaningful results.

Nevertheless, a few problems appeared in this study, some of which can be omitted in the future, whereas others are in the nature of the system and cannot be avoided.

- Some of the data from different experiments did not agree with each other. For example, the root K concentration in control Arabidopsis plants obtained from the 25 h salt treatment study was lower than measured in the kinetic analysis.
- Large standard deviation occurred for some ions, for example, the shoot Ca and Mg concentrations in plants after long-term salt treatment.
- Not exactly the same experimental conditions were used for some of the comparisons. For example, in the comparison between net and unidirectional Na uptake, unidirectional Na influx was measured under steady-state conditions but net Na uptake was not.
- The microarray analysis of ion transporter expression had several shortcomings. For example, the respective mRNA samples from Arabidopsis and Thellungiella were not hybridized to the same array. The efficiency of hybridization to the Arabidopsis array might be different for mRNA samples from Arabidopsis and Thellungiella due to the differences in the cDNA sequences. The same problem would apply to any PCR based quantification as sequence mismatch between

primers and target would lead to inefficient priming. Transcripts with lower signal in Thellungiella than in Arabidopsis can only be considered as being lower expressed if it is confirmed that the sequence of the targeted mRNA region matches the probe sequence. Another problem is that the normalization procedure decreases the actual fold change values of the transcripts; correction procedures can be applied but rely on certain assumptions (Cope et al., 2004). Finally, the Arabidopsis microarray can only analyse Thellungiella genes that have close homologues in the Arabidopsis genome, but not genes that are unique to Thellungiella.

- The difference in Na transport between Arabidopsis and Thellungiella might not be caused by differences of transporters at the transcriptional level.

A huge amount of data has been produced in this study, which provides important information for future physiological and molecular studies of both Arabidopsis and Thellungiella. In the following I will summarize and discuss the main results.

1. After salt stress Thellungiella accumulates less Na in the shoots than Arabidopsis. Net uptake of Na into both roots and shoots was slower in Thellungiella than in Arabidopsis. Similar results were obtained from comparing salt-sensitive and salt-tolerant rice cultivars and durum wheat landraces (Davenport et al., 2005; Kader and Lindberg, 2005). Hence, an increasing body of evidence suggests that low net Na accumulation during salt stress is a typical feature of salt tolerant plants.
2. Lower unidirectional Na influx into root cells is the main reason for the lower Na accumulation in Thellungiella than in Arabidopsis. To my knowledge this study provides the first direct evidence of low unidirectional Na influx into a halophyte using ^{22}Na. Kader and Lindberg (2005) demonstrated lower

unidirectional Na influx into roots of the salt-tolerant indica rice cultivar Pokkali compared to a salt-sensitive cultivar BRRI Dhan29 using a fluorescent sodium-binding dye (SBFI). Schubert and Läuchli (1990) also found lower unidirectional Na influx into roots of the salt-tolerant maize cultivar Pioneer 3906 compared to the salt-sensitive cultivar DeKalb XL75. Based on these findings I propose that the mechanisms underlying root Na influx rather than those underlying Na efflux (Shi et al., 2000) or Na compartmentation (Apse et al., 1999) should be at the centre of salt tolerance research. Indeed, it was revealed by my study that unidirectional efflux from roots is lower in Thellungiella than in Arabidopsis. This indicates that engineering of crops with enhanced Na efflux capacity might encounter problems (e.g. energy-requirement) that evolution has avoided. Increasing the capacity for Na compartmentation is probably a viable strategy, which however might not be sufficient unless supported by a low rate of Na uptake.

3. Voltage-independent cation channels (VICs) are likely to be the Na uptake pathway in both Thellungiella and Arabidopsis. VICs have been identified as a major Na uptake pathway in Arabidopsis and other glycophytes where they are non-selective among cations (Amtmann and Sanders, 1999; Davenport and Tester, 2000; Demidchik and Tester, 2002). Unidirectional Na influx into roots of Thellungiella followed similar kinetics as in Arabidopsis (Essah et al., 2003). Unidirectional Na influx into both species is inhibited by external Ca, but not by blockers of voltage-sensitive K channels (Essah et al., 2003). These features were also demonstrated for instantaneous currents across the plasma membrane of root protoplasts of Thellungiella by whole-cell patch-clamping (Volkov and Amtmann, submitted). The K/Na selectivity of the instantaneous currents in

Thellungiella root protoplasts is significantly higher than in Arabidopsis (Volkov et al., 2004). Because of the low Na permeability of the plasma membrane, Na-induced depolarisation of the membrane potential of root cells was less strong in Thellungiella than in Arabidopsis (Volkov and Amtmann, submitted). The values for Na inward currents measured at the respective resting potentials in 100 mM NaCl corresponded to the values for unidirectional Na influx measured in this study, both relatively (2× higher in Thellungiella than in Arabidopsis) and absolutely (Volkov and Amtmann, submitted). Therefore VICs are very likely to be the main Na uptake pathway in both species but appear to have species-specific ion selectivity properties.

4. Microarray analysis was used to compare the expression of ion transporter genes between Arabidopsis and Thellungiella. After salt stress both species showed a tendency to reduce Na uptake by decreasing the expression of possible pathways for Na influx. However, transcriptional control of putative Na transporters occurred in Arabidopsis in the shoots, whereas it occurred in Thellungiella in the roots. These results imply that ion transporters are at least partly regulated at transcriptional level during the plants' responses to high salt.
5. CNGC8 is a likely candidate for a Na uptake pathway in both Arabidopsis and Thellungiella. Transcript levels of CNGC8 decreased during salt stress in Thellungiella roots and Arabidopsis shoots. Other members of the Arabidopsis CNGC family have been shown to mediate K or Na uptake, and are sensitive to external Ca, which agrees with the characteristics of voltage-independent channels (Davenport and Tester, 2000; Demidchik et al., 2002; Demidchik and Tester, 2002; Tyerman, 2002). Therefore the cloning and functional

characterisation of this channel from both species is an urgent task for future research in this area.

In conclusion several interesting questions have arisen from this study.

- If Na accumulation is limited, how does Thellungiella adjust its osmotic potential during salt stress?
- Is the difference in root Na uptake the result of differences in protein structure, gene expression or post-translational modification of ion transporters between the two species?
- Is the observed difference in Na uptake necessary and/or sufficient for salt tolerance?

To address the first question the osmotic potential and turgor as well as the concentrations of potential organic osmolytes need to be measured in both species. An initial study by Inan et al. (2004) showed hyper-accumulation of proline in Thellungiella which could function as a compatible osmolyte. A more comprehensive metabolite analysis (Gong et al., 2005) confirmed a large increase in proline level after salt stress in Thellungiella. An increase in shoot concentrations was also found for other amino acids e.g. glutamic acid. Interestingly a variety of organic and inorganic solutes were present in much higher concentrations in Thellungiella than in Arabidopsis, among which glucose, a sugar similar to trehalose and sugar alcohols (inositol and galactinol) were further up-regulated, sucrose, organic acid (citric acid and malic acid) and inorganic phosphate were down-regulated, and fructose, sorbase and succinic acid were not changed by salt (Gong et al., 2005). The first group of the listed solutes are good candidates for replacing Na in its function as an osmolyte.

To answer the second question methodological approaches are required that fill the gap between the gene expression data and the ion transport data. In fact, a proteomics study

was initiated as part of my PhD project. Protocols for protein isolation and separation on 2-D gels were optimized for Thellungiella and a preliminary analysis of protein samples from control and salt-stressed plants proved that this is a feasible approach for studying salt-regulation at the protein level. However relatively few proteins encoding membrane transporters were present on the gels and alternative approaches (e.g. membrane fractionation or ICAT) should be investigated for their suitability for this aspect of the research. Useful hints for future studies could also be obtained by investigating the effect of a range of inhibitors (e.g. inhibitors of protein synthesis, phosphatases/ kinases and other known regulatory elements) on Na uptake.

To address the last question (proof of salt tolerance determination) a current project in the Glasgow laboratory uses a micrografting approach (Turnbull et al., 2002). Arabidopsis shoots are grafted onto Thellungiella roots and *vice versa*, allowing the researcher to evaluate (i) whether low Na uptake by the Thellungiella root system is sufficient to confer salt tolerance to plants with Arabidopsis shoot features and (ii) whether high Na uptake by the Arabidopsis root system is sufficient to confer salt sensitivity to the plants with Thellungiella shoot features. So far only one type of graft has been achieved combining Arabidopsis roots with Thellungiella shoots, and physiological experiments to characterize salt tolerance in these plants are in progress.
Another line of research deriving from this question exploits the low Na uptake background in Thellungiella for identifying genes from glycophytic species (e.g. Arabidopsis and wheat) that increase Na uptake in Thellungiella. A number of genes (including several CNGCs, HKT1 and LCT1) are currently under investigation in the Glasgow laboratory applying both transient and stable transformation protocols from Arabidopsis to Thellungiella.

A vigorous proof that differential Na uptake is a salt tolerance dominant would nowadays be sought through a mutagenesis approach. However, this requires further knowledge of the molecular mechanisms underlying low Na uptake in Thellungiella. If low Na uptake is due to structural features of a particular transport protein knocking out this gene would not result in salt sensitivity nor would over-expression of it increase salt tolerance. In this case one would seek to alter functional domains in the protein controlling its ion selectivity. By contrast, if a negative regulator is involved in the down-regulation of a Na pathway, knock out (or knock down by RNAi) would be a reasonable strategy to prove the roles of the regulator and the pathway in salt tolerance. Last not least, candidate genes (e.g. CNGC8) for Na uptake should be cloned from Thellungiella, their expression patterns and membrane localisation determined *in planta* and their functional mechanisms analyzed in heterologous expression systems.

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