Untersuchung zur Salztoleranz der Halophyten *Aster tripolium* L. und *Sesuvium portulacastrum* L. mit Hilfe von physiologischen, biochemischen und molekular-biologischen Methoden

Dem Fachbereich Biologie der Universität Hannover

zur Erlangung des Grades

Doktor der Naturwissenschaften

Dr. rer. nat.

genehmigte Dissertation

von

M.Sc. Balasubramanian Ramani

geboren am 13.03.1977
in Agraharam, Tiruchirapalli, Indien

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Investigation of salt tolerance mechanisms in the halophytes *Aster tripolium* L. and *Sesuvium portulacastrum* L. through physiological, biochemical, and molecular methods

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to my beloved elder Sister

Referent: Prof. Dr. Ahlert Schmidt
Koreferent: Priv.-Doz. Dr. Hans.-Werner Koyro
Tag der Promotion: 08.04.2004

Summary

The objective of this work was to use *Aster tripolium* L., which might be used as a vegetable in Europe, and *Sesuvium portulacastrum* L., a vegetable and fodder plant in South Asia, to investigate the salt tolerance mechanisms in these halophytes. To compare the strategies involved in salt tolerance of two different halophytes *Aster tripolium* (Dollart, Germany) and *Sesuvium portulacastrum* (Dakhla, Morocco) were grown with 1.5%/257 mM and 3%/513 mM or 2.5%/428 mM, 3%/513 mM, and 5%/856 mM NaCl, respectively, in the watering solution for upto 10 days. The quotient of Na^+/K^+ indicates that *Aster* accumulates more K^+ in comparison to Na^+ while the reverse is true for *Sesuvium*. The CO_2 assimilation rate decreased in *Aster* (3%/513 mM) and *Sesuvium* (5%/856 mM) NaCl-treated plants significantly at the end of day 10. Maximum electron transport rate was not affected but non-photochemical quenching increased with increasing NaCl concentrations in both *Aster* and *Sesuvium*. P-ATPase decreased and F-ATPase increased with increasing NaCl in *Aster* whereas V-ATPase activities were unaffected in both the plants. Plant sulfolipids (SQDG-Sulfoquinovosyldiacylglycerol) are anionic glycerolipids localized in the thylakoid membranes constituting 6-7% of total lipids. A modified method for the reproducible quantification of sulfolipids in plant lipid extracts by thin layer chromatography was established. Sulfolipid content increased in both of these halophytes investigated, and slightly in *Thellungiella halophila* O. E. Schulz, a close salt tolerant relative of *Arabidopsis thaliana* Heynh., but not in *Arabidopsis* (ecotype Wassilewska). Using LC-MS, molecular species identification has been made of fatty acids composition which showed different identities. The impairment of sulfur metabolism in salt treatment was investigated through enzymatic analysis of β-cyanoalanine synthase (EC 4.4.1.9) and cysteine synthase (EC 4.2.99.8), which were significantly affected due to salt stress in *Aster* leaves and roots. Plasma membranes (PM) were isolated from *Aster* plants grown at 0% and 3% NaCl and purity was verified by sequencing and alignment. The transport activity of *Aster* H^+-ATPase was measured in characterized PM vesicles and to be found similar in untreated (0% NaCl) and salt-treated (3% NaCl) plants. 2D gel electrophoresis and results of sequencing using mass spectrometry methods revealed differences in the presence and abundance of several proteins in *Aster* plants watered with NaCl solutions. The microarray technology was used to investigate the transcriptome variation in *Aster* plants treated with 0% and 3% NaCl. Correlating all the above results, strategies to use *Aster tripolium* and *Sesuvium portulacastrum* as crop plants are discussed. **Keywords:** *Aster tripolium* L., *Sesuvium portulacastrum* L., ATPase activity, 2D gel electrophoresis, microarray, sulfolipids.

Zusammenfassung

Das Ziel dieser Arbeit war es, die Salztoleranz-Mechanismen der halophytischen Pflanzen *Aster tripolium* L., die in Europa als Gemüse verwendet wird, und *Sesuvium portulacastrum* L., die in Südasien als Gemüse- und Futterpflanze genutzt wird, zu verstehen. Um die Strategien dieser beiden verschiedenen Halophyten in Bezug auf Salztoleranz vergleichen zu können, wurden *Aster tripolium* (Dollart, Deutschland) und *Sesuvium portulacastrum* (Dakhla, Marocco) mit 1.5%/257 mM und 3%/513 mM bzw. 2.5%/428 mM, 3%/513 mM und 5 %/856 mM NaCl im Gießwasser angezogen. Der Quotient aus Na^+/K^+ zeigte, dass *Aster* mehr K^+ im Vergleich zu Na^+ akkumulierte, während für *Sesuvium* das Gegenteil festgestellt wurde. Die CO_2-Assimilationsrate nahm in NaCl-behandelten *Aster*-(3%/513 mM) und *Sesuvium*-Pflanzen (5%/856 mM) bis zum Tag 10 des Versuchs ab. Bei der Untersuchung von kurzfristigem Salzstress auf die P-, F-, und V-ATPase zeigte sich für *Sesuvium* keine Aktivitätsänderung, während bei *Aster* eine Abnahme der P-ATPase und eine Zunahme der F-ATPase Aktivität festgestellt wurde. Pflanzliche Sulfolipide sind in der Thylakoidmembran lokalisierte anionische Glycerolipide, welche 6-7 % der gesamten Lipide ausmachen. Eine modifizierte Methode zur reproduzierbaren Quantifizierung von Sulfolipiden in Pflanzenextrakten mittels Dünnschichtchromatographie wurde etabliert. In beiden untersuchten Halophyten stieg der Sulfolipidgehalt an. In *Thellungiella halophila* O. E. Schulz, einer salztoleranten nahen Verwandten von *Arabidopsis thaliana* Heynh., aber nicht in *Arabidopsis* (Ökotyp Wassilewska), stieg der Sulfolipidgehalt ebenfalls an. Die Fettsäuremuster der Sulfolipide wurden mittels LC-MS analysiert und Veränderungen bei Salzbehandlung festgestellt. Eine mögliche Beeinträchtigung des Schwefelstoffwechsels bei Salzbehandlung wurde durch enzymatische Analyse der β-Cyanoalaninsynthase (EC 4.4.1.9) und Cysteinsynthase (EC 4.2.99.8) untersucht; beide Enzyme wurden in Blättern und Wurzeln signifikant durch Salzstress beeinflusst. Plasmamembranen (PM) von *Aster*-Pflanzen, gegossen mit 0 und 3% NaCl wurden isoliert und charakterisiert durch Messung der Transportaktivität der *Aster*-H^+-ATPase in isolierten PM-Vesikeln, wobei die Ergebnisse in unbehandelten und mit NaCl behandelten (3%) Pflanzen ähnlich waren. Zweidimensionale Gelelektrophorese und eine Sequenzanalyse mittels Massenspektroskopie ergaben Unterschiede in Vorkommen und Häufigkeit verschiedener Proteine. Die Microarray-Technologie wurde angewandt, um Aufschluß über Transkriptomveränderungen durch Salzbehandlung zu bekommen. Unter Einbeziehung aller Ergebnisse werden Möglichkeiten zur Nutzung von *Aster tripolium* und *Sesuvium portulacastrum* als Nutzpflanzen diskutiert.

Schlüsselwörter: *Aster tripolium*, *Sesuvium portulacastrum*, ATPase-Aktivität, 2D-Gelelektrophorese, Microarray, Sulfolipide.

Abbreviations

1D	One dimensional
2D	Two dimensional
AAS	Atomic absorption spectrometry
ATP	Adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolylphosphate
BPB	Bromophenolblue
BSA	Bovineserumalbumin
CAS	β-cyanoalaninesynthase
CHAPS	3[3-Chloromidopropyl)Dimethyl-Amino]-1-Propansulfonate
Coomassie	Coomassie Brilliant Blue R-250
DAG	Diacylglycerol
DGDG	Digalactosyldiacylglycerol
DNA	Deoxyribonucleicacid
EDXA	Energy dispersive X-ray analysis
GC-MS	Gas chromatography – mass spectrometry
g	Earth accelaration
HPLC	High pressure liquid chromatography
IEF	Isoelectric focussing
IPG	Immobilised pH-Gradient
LC-MS/MS	Liquid chromatography coupled with mass spectrometry
MALDI-TOF-MS	Matrix-assisted laser desorption time-of-flight mass spectrometry
MES	2-(N-Morpholino)ethanesulfonicacid
MOPS	2-(N-Morpholino)propanesulfonicacid
MGDG	Monogalactosyldiacylglycerol
NBT	Nitrotetrazoliumbluechloride
NPQ	Non-photochemical quenching
OAS-TL	O-acetylserine (thiol)-lyase
PAGE	Polyacrylamide-gel electrophoresis
pI	Isoelectric point
PPFD	Photosynthetic photon flux density
PQ	Photochemical quenching
PSII	Photosystem II
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase-polymerase chain reaction
SEM	Scanning electron microscope
SDS	Sodiumdodecylsulfate
SQDG	Sulfoquinovosyldiacylglycerol

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1 GENERAL INTRODUCTION

1.1 Agricultural productivity affected by salinity and salinization

Population growth, changes in lifestyle, competition for fresh water between farmers and cities, and possible global environmental changes have led to alarming projections that seem to argue additional strategies by which food supply can be guaranteed (Miflin, 2000). Soil salinity is a major constraint to food production because it limits crop yield and restricts use of land previously uncultivated. The constraints are more acute in areas of the world where food distribution is problematic because of insufficient infrastructure or political instability. Large areas of the Indian subcontinent, China's North Plain, Australia, Central Asia, parts of the Canadian prairies, the United States, Mexico, Paraguay, and Uruguay are all severely affected (Owens, 2001; Essah, 2002). Historically arid regions in Africa and the Middle East are expanding, and shortages of freshwater are appearing in places, such as the Asia-Pacific rim and Northeast Brazil, that once never doubted their water supplies (Moffat, 2002). Salinity affects 7% of the world's land area, which amounts to 930 million ha (Szablocs, 1994). The area is increasing, a global study of land use over 45 years found that 6% had become saline (Ghassemi et al., 1995). The intensity of affected agricultural areas due to salinity was shown in figure 1. This amounts to 77 million ha, despite its relatively small area; irrigated land is estimated to produce one-third of the world's food (Munns, 2002). Figure 2 shows the land area still suitable for agriculture around the world. Productivity will need to increase by 20% in the developed world and by 60% in the developing world and so the best possible use must be made of these once productive soils (Owens, 2001).

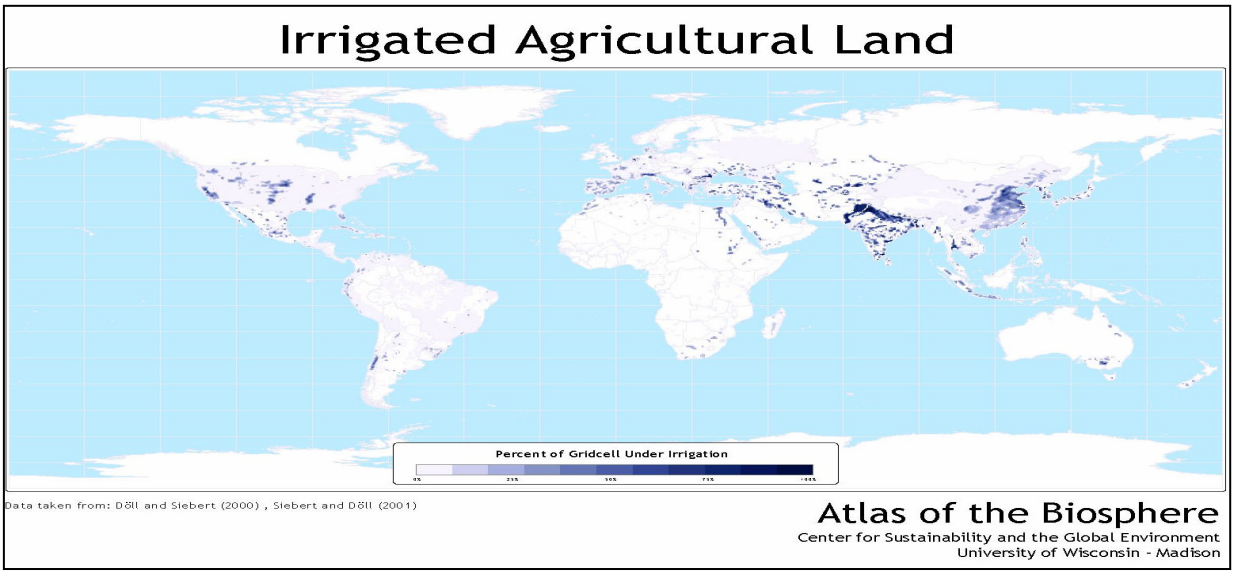


Fig. 1 This world atlas shows the intensity of affected agricultural areas due to salinity (Siebert and Döll, 2001). Percent of gridcell shows land still under irrigation.

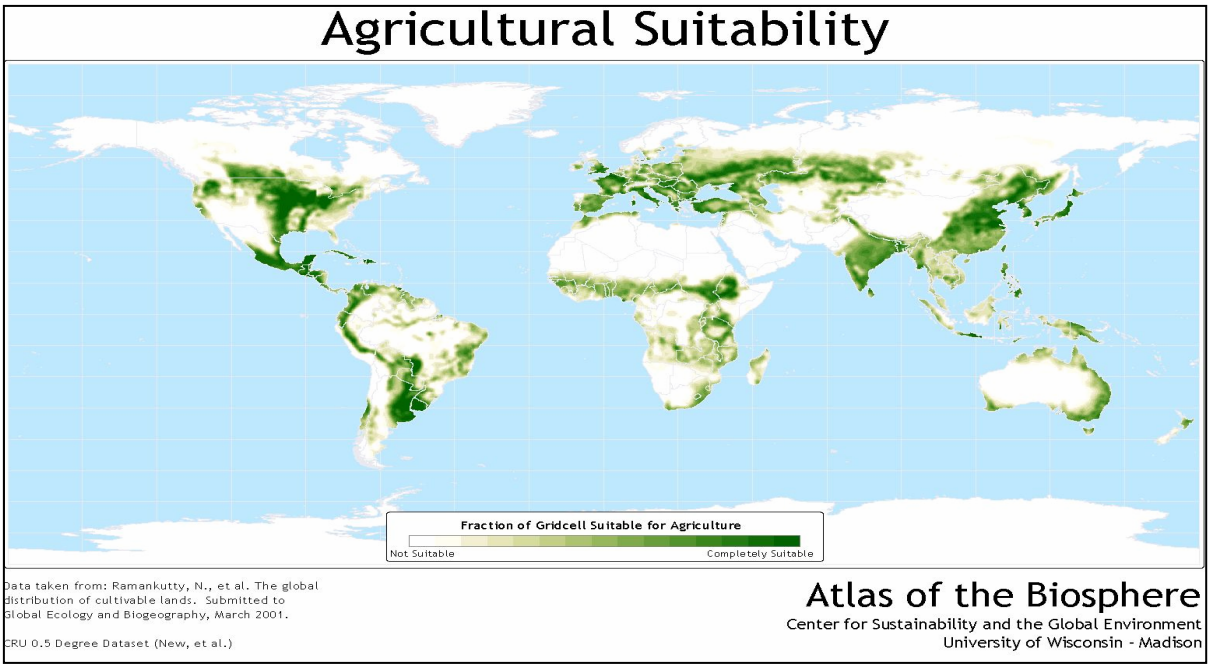


Fig. 2 This world atlas shows the land area still suitable for agriculture (Ramankutty, 2001). Percent of gridcell shows land still suitable of agriculture.

Plant growth and development depend on the uptake, translocation and sorting of at least 15 essential nutrients and countless metabolites to specific organs, cells and subcellular compartments (Sze et al., 2002). From an agricultural point of view, the accumulation of salts to excessive levels in the rhizosphere results in salinity (Gorham, 1992). Salinity can take two forms. Primary salinity is due to 'natural' causes such as salt in a sea spray being carried inland by prevailing winds and deposited by rainfall, the periodic submergence of land under seawater, and also the process of weathering which can gradually lead to the accumulation of salts in soil water. Secondary salinisation can develop due to the policy of land clearing which raises groundwater levels and brings salt to the surface, and the problems of deforestation and overgrazing by animals. However, secondary salinisation stems most often from the over-irrigation of land, which is ironic given that this commonly occurs in arid and semi-arid zones where water supply is a problem (Essah, 2002).

To plant life, salinity is just one inimical factor of the environment. To man, salinity creates a problem due to its effects on his crop species which are predominantly sensitive to the presence of high concentrations of salts in the soil. In addition, crop plants must be capable of satisfactory biomass production in a saline environment (yield stability). Tolerance and yield stability are complex genetic traits that are difficult to establish in crops since salt stress may occur as a catastrophic episode, be imposed continuously or intermittently, or become gradually more severe, and at any stage during development. To overcome these

limitations and improve production efficiency in the face of a burgeoning world population, more stress tolerant crops must be developed (Khush, 1999). The agricultural problem of salinity tolerance is probably best tackled by either altering farming practices to prevent soil salinization occurring in the first place, or by implementing schemes to try to remediate salinized soils (such as by planting perennials to lower water tables) (Tester and Davenport, 2003).

As salinity stress is a continuing and increasingly deleterious obstacle to the growth and yield of crop plants, owing to irrigation practices and increasing demands on fresh water supply, the engineering of salt tolerant crop plants has been a long-held and intensively sought objective (Apse and Blumwald, 2002).

1.2 Need for halophytes as experimental plants

Halophytes are plants that have been naturally selected in saline environments and are distinguishable from glycophytes by their capacity to cope with excessive levels of ions with high proficiency (Greenway and Munns, 1980; Flowers et al., 1986). One of the least successful areas of crop science has been the search for salt-tolerant cultivars (Kerstiens et al., 2002). Traditionally, studies directed toward understanding salt tolerance have investigated the response of glycophytes (plants that are salt sensitive) to salt stress (Parks et al., 2002). Glenn et al. (1999) pointed out that most of the research has been conducted upon our existing range of crops, nearly all of which originate from glycophytes that appear to lack any genetic basis for salt tolerance. They argued that the focus of attention in future should be upon halophytes, to discover how they are able to maintain production under saline condition- the aim being to ‘define a minimal set of adaptations required in tolerant germplasm’. This is by no means a simple objective because halophytes display huge diversity, having apparently evolved independently in many different families and genera (Kerstiens et al., 2002). It is now considered important to discover any particular traits that are so essential for salt tolerance that they appear in many different halophytes as a result of convergent evolution (Glenn et al., 1999). Studies have focused on comparisons of ion-transporting activities of closely related species grown in the presence of high NaCl concentrations (Staal et al., 1991; Wilson and Shannon, 1995) and physiological parameters (water potential, turgor pressure, photosynthesis, respiration, or ion content) in plants grown with and without salt stress (Gale et al., 1967; Hoffman et al., 1980; Erdei and Teleisnik, 1993). While these studies provide evidence that genetic variability for salt tolerance exists, the actual mechanisms underlying salt tolerance are not well understood. An alternative approach is to make similar comparative

measurements with halophytes which grow optimally in elevated levels of Na^+ (Flowers et al., 1977). Such species can be grown in high or low Na^+ concentrations, making it possible to compare growth and physiological parameters at different salinity levels without the confounding effects of Na^+ toxicity.

1.3 Sodium versus potassium during salt stress

For plants Na^+ and K^+ are the most important of the group1 cations and although Na^+ dominates the hydrosphere, neither predominates in the lithosphere. Although we cannot measure the absolute amounts of the two elements that are available to plants, we can gain some indication by comparing their concentrations in soil solutions: K^+ concentrations range from 0.2 to 10 mM (about 1 to 2 mM) while Na^+ concentrations lie between 0.4 to 150 mM. The only real distinction between the two elements in this respect is the occurrence of very high Na^+ concentrations, and these can generally be traced back either to the influence of seawater or to the results of irrigation in areas of high evapotranspiration (Flowers and Läuchli, 1983). The sensitivity of cytosolic enzymes to salt is similar in both glycophytes and halophytes, indicating that the maintenance of a high cytosolic K^+/Na^+ concentration ratio is a key requirement for plant growth in salt (Glenn et al., 1999). As well as dealing with Na^+, the cell must also acquire nutrient K^+. Also, high and constant physiological concentrations of K^+ (100-200 mM), along with low concentrations of Na^+ (1-30 mM) are required in the cytoplasm for normal cytoplasmic processes (Koyro and Stelzer, 1988; Binzel et al., 1988 ; Carden, 1999). Na^+ concentrations above 100 mM or low K^+ to Na^+ ratios can inhibit special functions in enzymes through the ability of Na^+ to compete with K^+ for K^+ binding sites (Greenway and Munns, 1980; Gorham et al., 1985; Tester and Davenport, 2003). High Na^+ levels can also interfere with cytosolic enzyme function because whereas K^+ is an essential activator of more than 50 enzymes, Na^+ is unable to substitute for K^+ in this role (Bhandal and Malik, 1988). Interestingly, cytosolic enzymes in halophytes are also not adapted to high salt levels, and display the same sensitivity to salt as enzymes from glycophytes (Flowers et al., 1977). Na^+ may also cause disruption of cytoplasmic components such as microtubules, microfibrils, spherosomes, and ribosomes (Mansour et al., 1993). Salt accumulation in the shoot can reduce plant photosynthetic leaf area through Na^+ inhibition of cell division and expansion and Na^+-induced leaf senescence, all of which decrease photosynthetic efficiency and contribute to ever-decreasing plant growth and productivity, and sometimes even death (Munns and Termaat, 1986; Munns, 1993; Yeo, 1998). Plants depend upon K^+ to regulate the opening and closing of stomata, enzyme activation, protein synthesis, photosynthesis,

osmoregulation, cell extension, phloem transport and cation-anion balance. When K^+ moves into the guard cells around the stomata, the cells accumulate water and swell, causing the pores to open and allowing gases to move freely in and out, whereas when K^+ supply is inadequate, the stomata become sluggish and hence water vapor is lost (Beegle, 1988).

1.4 Photosynthetic parameters as a tool for salinity tolerance screening

The decline in growth observed in many plants subjected to excessive salinity is often associated with a decrease in their photosynthetic capacity (Long and Baker, 1986). This opens the possibility of using photosynthetic parameters to screen salt tolerant crops. The rationale for the view that changes in leaf photosynthetic parameters may be used to carry out screening of stress-resistant cultivars is that these parameters would reflect any constraint acting on the photosynthetic processes (Belkhodja et al., 1994).

1.4.1 Leaf gas exchange parameters and metabolic adaptations of plants to stress

Generally photosynthetic activity is suppressed under salt and water stress and it is severely suppressed in salt-sensitive species such as crop plants or glycophytes (Seemann and Critchley, 1985). The guard cells that form the stomatal pore control the flux of CO_2, H_2O, and other gases between the plant and the atmosphere and are regulated by both internal and external factors (Lawson et al., 2003). Although the physiological properties required for salt tolerance are complex, it is clear that stomatal control of gas exchange cannot be less important in halophytes than in glcophytes. It may indeed be of even greater consequence, because once water and solutes have passed any symplastic barrier in the roots of halophytes, their rate of upward passage in the xylem is largely determined by the rate of transpiration. This means that in addition to maintaining water balance, stomatal control can influence the salt content of the shoot even though the partial exclusion of salt by the roots is of paramount importance (Kerstiens et al., 2002). When we discuss factors involved, one should evaluate two points: “stomatal limitation” and “non-stomatal limitation”. Stomatal limitation means that the photosynthetic rate declines due to a reduction in the level of CO_2 supply as a result of stomatal closure. On the other hand, non-stomatal limitation occurs by a decrease in the activity of CO_2 fixation in the mesophyll cells because of water loss or excessive salts. It is important to recognize whether reduction of photosynthetic activity is caused by stomatal closure or damage to the activity of RubisCO and Calvin cycle enzymes and/or light reaction activity under stress conditions (Ueda et al., 2003).

It has been suggested that decreases in growth with salinity may be due to increased respiration rates resulting from higher energy requirements (Gale, 1975; Schwarz and Gale, 1981). Others have attributed the depression in growth rates to the combined effects of salinity on photosynthesis and the pattern of carbon allocation (De Jong, 1978a, b). The photosynthetic rate (*A*) of leaves of both C_3 and C_4 plants decreases as their relative water content (RWC) and water potential (Ψ) decrease (Cornic, 1994; Kramer and Boyer, 1995; Lawlor, 1995). Stomatal limitation is considered to decrease both the photosynthetic rate *A* and the CO_2 concentration in the intercellular spaces of the leaf (C_i), which inhibits metabolism (Kaiser, 1987; Downton et al., 1988).

Gas exchange parameter will give us very clear signal on the effect of stress when investigating plants switching from C_3 to CAM. Crassulacean acid metabolism (CAM) plants are dependent on the limited supply of malate that is accumulated overnight in the vacuoles as a source of CO_2 during the daylight deacidification period, when stomata are closed and high irradiances generally prevail (Franco et al., 1999). The transition from C_3 to CAM is under both developmental and environmental control (Lüttge 1993, Cushman and Bohnert, 1999). The transition of photosynthetic carbon assimilation pathway from C_3 to CAM have been studied mainly after salinity and drought stress (Lüttge 1993; Adams et al., 1998). It has also reported that light intensity and quality play a crucial role during C_3 to CAM shift (Grams and Thiel, 2002). Moreover, diurnal fluctuations in C_4-acid concentration (mainly malic acid) may also be indicative of the appearance of CAM (Cushman and Bohnert, 1999).

1.4.2 Effect of salt stress on osmolarity, osmotic potential and osmolytes

The immediate effect of salt and water stress can be seen in the changes to osmolarity, leaf water potential and accumulation of osmolytes to control the cell turgor pressure. Metabolic acclimation via the accumulation of compatible solutes is regarded as a basic strategy for the protection and survival of plants in extreme environments (Sakamoto and Murata, 2000). Compatible solutes have been defined as organic osmolytes responsible for osmotic balance and at the same time compatible with cell's metabolism (Galinki, 1993). Metabolites that serve as compatible solutes differ among plant species and include polyhydroxylated sugar alcohols, amino acids and their derivatives, tertiary sulphonium compounds and quaternary ammonium compounds (Bohnert and Jensen, 1996).

The injury suffered by plants exposed to sodium chloride is considered to be the result of both ionic and osmotic damage due to a lowered water potential (Gale, 1975; Levitt, 1980). The two factors are interrelated and coexist under saline conditions. Halophytes must possess

the ability to counter both stresses simultaneously (Kefu et al., 2003). Osmotic stress can occur in the leaf apoplast, and this mechanism of Na^+ toxicity was first proposed by Oertli (1968). High apoplastic Na^+ concentrations can induce a flux of water from the cells, causing a decrease in turgor and an increase in concentrations of intracellular solutes (Lichtenthaler, 1995). As water-generated turgor pressure is a driving force for cell expansion, the decrease in turgor could also result in reduced rates of cell expansion (Xiong and Zhu, 2002a). Summary of the functions of different components in protecting the cell during salt stress was shown in figure 3.

1.4.3 Chlorophyll fluorescence and pigments

In recent years, the technique of chlorophyll fluorescence has become ubiquitous in plant ecophysiology studies. Chlorophyll fluorescence has been shown to be a non-invasive, powerful, and reliable method for assessing photosystem II (PSII) function (Lu et al., 2003). The analyses of chlorophyll fluorescence quenching provide information on the fundamental processes of energy absorption, utilization, and dissipation of excess energy, and electron transport in PSII (Krause and Weis, 1991; Schreiber et al., 1994; Govindjee, 1995). The decrease in photosynthesis induced by salt stress enhances the amount of excess excitation energy (Björkman, 1989), which, if not safely dissipated, may result in photodamage to PSII because of an over-reduction of reaction centers and subsequent formation of active oxygen species (Demmig-Adams and Adams, 1992). Although much efforts have been invested to elucidate the causes of the decreased photosynthetic capacity, the underlying mechanism are still unclear. The data on the effects of salinity stress on PSII photochemistry are conflicting. Some studies have shown that salt stress inhibits PSII activity (Masojidek and Hall, 1992; Belkhodja et al., 1994), whereas other studies have indicated that salt stress has no effect on PSII (Robinson et al., 1983; Brugnoli and Björkman, 1992; Morales et al., 1992).

Changes in the composition and function of the photosynthetic apparatus of plants in response to salinity constitute an important parameter to be analysed. For, instance some changes in photosynthetic pigment composition, such as decrease in neoxanthin and increases in zeaxanthin at the expense of violaxanthin, have been reported in salt stressed sorghum (Sharma and Hall, 1992). In this xanthophylls cycle, excess excitation energy can be harmlessly dissipated in the antennae complexes of PSII as heat through the formation of zeaxanthin by de-epoxidation of violaxanthin, via the intermediate antheraxanthin. Zeaxanthin and antheraxanthin are both thought to be involved in the photoprotective dissipation process

and are able to trap surplus excitation energy in the antennae complexes of PSII and to dissipate it harmlessly as heat (Demmig-Adams and Adams, 1992; Gilmore, 1997).

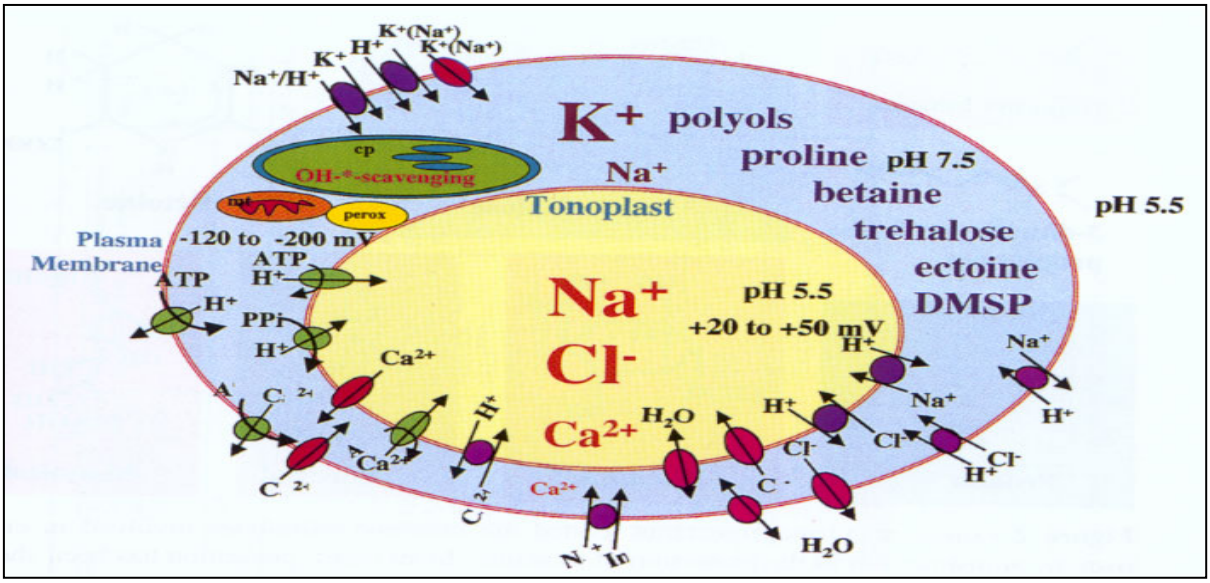


Fig. 3 This figure shows the function of different components in protecting the cell during salt stress (Hasegawa et al., 2000). Compartmentation of Na^+ and Cl^- in the vacuole, accumulation of osmolytes, Na^+/H^+ antiporters and scavenging of oxygen radicals.

1.5 Putative role of sulfolipids in higher plants

Plants contain a unique sulfoglycolipid, having a sulfonic acid C-SO_3H group and hence the name sulfolipid. In 1959, Benson and his co-workers discovered this new sulfur-containing lipid in plants and identified it as sulfoquinovosyldiacylglycerol (SQDG) (Benson, 1963). The distinctive feature of this substance with the structure of 1,2-di-O-acyl-3-O-(6-deoxy-6-sulfo-α-D-glucopyranosyl)-sn-glycerol is a carbon directly bound to sulfur as C-SO_3^-. Sulfonic acid of this type is chemically stable and a strong acid in a wide pH range (Barber and Gounaris, 1986). Sulfoquinovose and its glycosides, glyceryl sulfoquinovoside and SQDG are the most abundant anionic sugar derivatives in plants under various conditions (Okanenko, 2000).

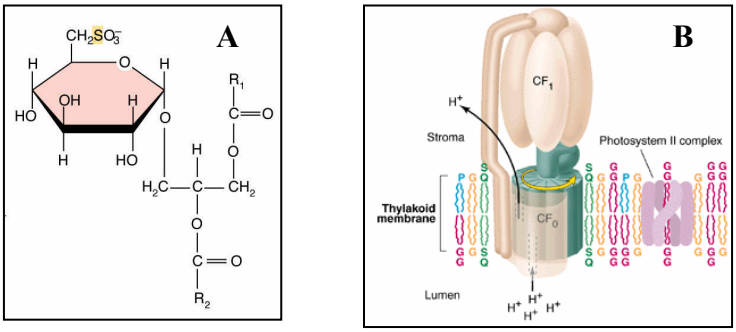


Fig. 4 A) The structure of the plant sulfolipid 1,2-di-O-acyl-3-O-(6-deoxy-6-sulfo-α-D-glucopyranosyl)-sn-glycerol (SQDG) showing the direct attachment of carbon to sulfur in the sulfoquinovose head group (Buchanan et al., 2000) and **B**) the close association of SQDG with the plastidic ATPase complex (Sharkey, 2000).

SQDG has been found in all photosynthetic plants, algae, cyanobacteria, purple sulfur and non-sulfur bacteria (Barber and Gounaris, 1986), non-photosynthetic bacterium *Rhizobium meliloti* (Cedergreen and Hollingsworth, 1994). SQDG is localized in the thylakoid membranes, being the most saturated glycolipid (Janero and Barrnett, 1981). None of other lipids can compete with SQDG in its quantity in the biosphere (Heinz, 1993). SQDG comprises between 8-24% of the four major chloroplast lipids and contains a substantial quantity of high melting fatty acids (16:0+18:0), the range lying between 26 to 62% (Kenrick and Bishop, 1986). SQDG appears to be concentrated in the chloroplast lamellar membranes of plants and was found in the outer and inner thylakoid membranes, both in the appressed and non-appressed region (Barber and Gounaris, 1986) and also digramatically represented in figure 4a and 4b. SQDG is also present in the outer and inner envelope membrane in amounts near 6% (Douce et al., 1984). In chloroplasts, the SQDG biosynthesis is catalysed by two enzymes namely SQD1 (sulfoquinovosyltransferase 1) and SQD2 (sulfoquinovosyltransferase 2). SQD1 catalyses the incorporation of SO_3^- into UDP-Glucose forming a head group UDP-sulfoquinovose. SQD2 catalyses the second and final reaction of SQDG formation by addition of DAG (diacylglycerol) into the head group as shown in figure 5.

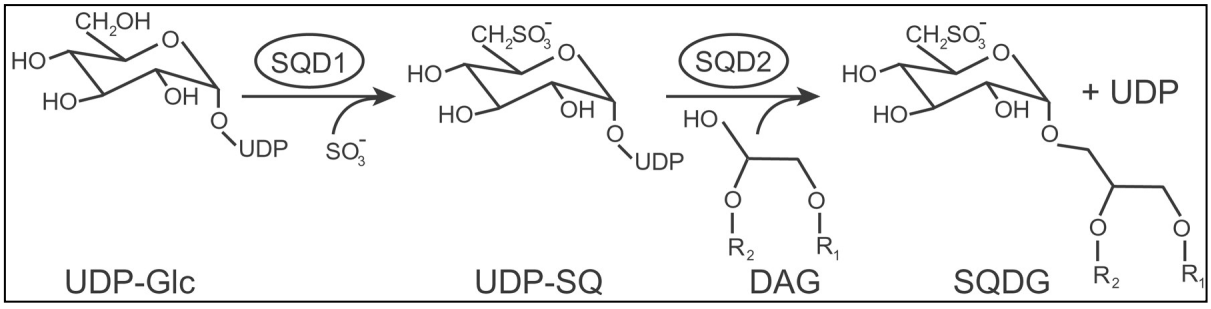


Fig. 5 The pathway for sulfolipid biosynthesis in *Arabidopsis*. Two enzymes, SQD1 and SQD2, are specific to this process and catalyze the reactions as indicated. DAG, diacylglycerol; R, fatty acyl groups; SQDG, sulfoquinovosyldiacylglycerol; UDP-Glc, UDP-glucose; UDP-SQ, UDP-sulfoquinovose (Yu et al., 2002).

Probable functions of plant sulfolipids include orientation of chlorophyll molecules in membranes (Benson, 1963; Anderson, 1975; Trosper and Sauer, 1968), has been suggested to have close association with protein complex of thylakoid membrane, high resistance of some SQDG molecules bound to CF_0-CF_1 suggesting exchange with other acidic lipids, also SQDG removal from CF_0-CF_1 ATPase complex inactivated it therefore to the assumption SQDG is an integral part of the protein complex (Pick et al., 1985). Environmental factors also have played the role in SQDG buildup like temperature (Pearcy, 1978), water deficit (Quartaci et al., 1995), but mixed reports on the effect of salinity (Müller and Santarius, 1978; Kuiper et al., 1974), also there is another recent hypothesis that sulfolipid can function as a substitute of anionic phospholipids under phosphate-limited growth conditions. Out of all probable and hypothesized functions of SQDG, there is no solid proof for any of the results so far to give a possible functional role to SQDG in plants.

1.6 Effects of NaCl on β-cyanoalanine synthase and cysteine synthase activities

β-Cyanoalanine synthase (CAS; EC 4.4.1.9) is widely distributed in higher plants and plays a pivotal role in cyanide detoxification fixation (Miller and Conn, 1980). CAS catalyses the conversion of cyanide and cysteine to β-cyanoalanine (Maruyama et al., 2000). The main physiological role of CAS has been suggested to be the detoxification of cyanide produced in various stages of the plant's life cycle (Hendrickson and Conn, 1969). CAS has been purified from some plants (Akopyan et al., 1975; Ikegami et al., 1988a; Ikegami et al., 1988b; Ikegami et al., 1989), and is believed to be exclusively compartmentalized in mitochondria (Akopyan et al., 1975; Wurtele et al., 1985). However, some researchers found that activity exists in cytosol (Wurtele et al., 1985; Cooney et al., 1980; Ikegami et al., 1989; Hasegawa et al., 1995). Cysteine synthase (*O*-acetyl-L-serine (thiol) lyase, EC 4.2.99.8) catalyses the formation of cysteine from O-acetylserine and bisulphide. This reaction is responsible for the incorporation of inorganic sulphur into the aminoacid cysteine, which can be subsequently converted into other sulphur-containing compounds, such as methionine or incorporated into the tripeptide glutathione (Warrilow and Hawkesford, 1998). Studies show that cysteine synthase expression can be influenced by nutritional status suggest that it may have a role to play in determining the amount of sulphur to be assimilated into the amino acid pool (Warrilow and Hawkesford, 1998). The final step of csyteine synthesis is catalysed by cysteine synthase (CS), or O-acetylserine (thiol)lyase, which transfers sulfide to O-acetylserine and releasing acetate (Nakamura.et al., 1999). The regulation of cysteine

synthesis is complicated by the fact that CS is regulated not only by sulfur, but also by nitrogen availability (Takahashi and Saito, 1996) and light (Kitamura et al., 1996). Cysteine synthase activity in higher plant systems reacts only slowly to changing environmental factors such as sulphate or nitrogen availability (Brunold and Schmidt, 1976; Brunold and Schmidt, 1978), however response to plant development is evident (Schmutz and Brunold, 1982; von Arab and Brunold, 1986). Using inorganic sulfate from the soil, plants and bacteria synthesize a variety of sulfur compounds, including sulfur-containing amino acids, coenzymes and sulfolpids. Sulfur metabolism also seems to affect photosynthesis (Wykoff et al., 1998). These observations suggest, first a coordinated regulation of CS isoforms in different cellular compartments and second cross-talk among sulfur and nitrogen metabolism and photosynthesis (Nakamura.et al., 1999).

1.7 Role of plasma membrane proteins in higher plants

The plasma membrane (PM) (fig. 6) regulates the exchange of information between the cell and its environment. In plants, the PM has many additional specific functions such as cell wall assembly and the response to environmental factors, including both biotic factors, such as pathogens, and abiotic factors, such as changes in edaphic conditions (Smallwood *et al*., 1996). A consequence of this situation is that the PM protein composition exhibits high plasticity (Masson and Rossignol, 1995). Numerous biochemical mechanisms are modified at the PM level upon changes of environmental and internal factors (Santoni et al., 2000).

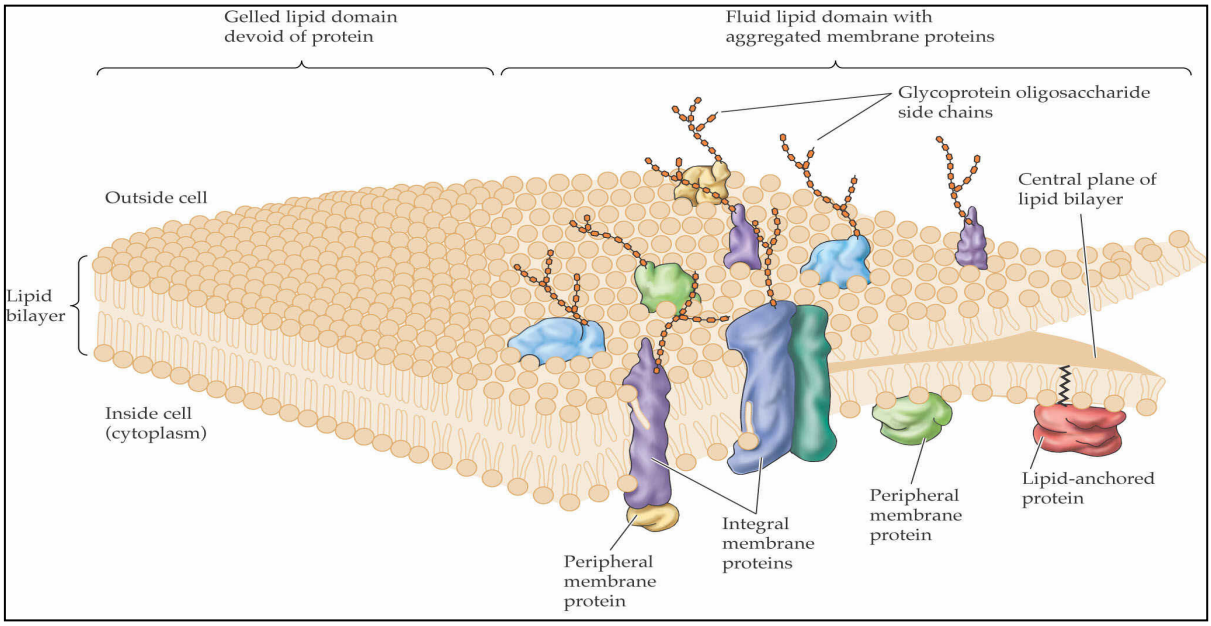


Fig. 6 A modern version of fluid-mosaic membrane model, depicting integral, peripheral, and lipid-anchored membrane proteins (Buchanan et al., 2000)

Identification of the subcellular localization of a particular protein provides essential information towards understanding its physiological function (Berczi and Asard, 2003). Despite considerable study on the nature of the plasma membrane of higher plants, little information is available regarding the molecular bases of the numerous physiological and/or biochemical functions of this important organelle. The nature of plasma membrane proteins has contributed to that situation. One reason for this is the intractable nature of plasma membrane proteins which makes them unamenable to analysis by standard biochemical techniques. The development of immobilized pH gradients (IPGs) for isoelectric focusing (IEF) has overcome the problems of gradient drift and low protein load capacity normally encountered with conventional carrier-ampholyte based separations (Bjellqvist et al., 1982). Therefore, two-dimensional electrophoresis is a key method in the current proteomics projects (Chevallet et al., 1998). Solubilization with various detergents, including sodium dodecyl sulfate (SDS), combined with one-dimensional or two-dimensional polyacrylamide gel electrophoresis has proved useful in a wide variety of microbial and mammalian studies; however, until recently those techniques have generally not been successful with higher plant plasma membrane proteins (Booz and Travis, 1981). Only a minority of proteins that ensure these various functions have been characterized to date, including in the model plant *Arabidopsis thaliana*. In fact, most of them correspond to isoforms of a few transport systems (Logan et al., 1997). In, addition, due to lack of specific sequences to identify PM proteins, very little direct information is to be expected from current systematic genome sequencing efforts (Santoni et al., 1999). However, little information is available concerning plasma membrane proteins and less than 10% are cloned (Sussman, 1994). Since genomic data from this organism are rare, molecular screenings with probes from known transporters can be used, but alternatively the molecular access to protein pattern is also enabled by 2D gel electrophoresis and subsequent mass spectrometry. Several enzymatic studies were performed with isolated plasma membranes. Plasma membrane preparations mainly contain sealed right-side-out (apoplastic side out) vesicles. Part of these vesicles have been turned inside-out by freezing and thawing, and sealed inside-out and right-side-out vesicles subsequently separated by repeating the phase partition step. The methods combine two aqueous polymer two-phase partitioning with sucrose density centrifugation (Larsson et al., 1987), and the purity of the membrane fractions has been verified by the use of antibodies against membrane specific markers.

1.8 Properties and putative role of plasma membrane (P-), vacuolar (V-) and during salt stress

It appears that one facet of membrane response to salinity is the differential response of ATPase to the salt stress. ATPases are integral transport proteins that hydrolyze ATP to pump protons across a membrane (Sze, 1985; Serrano, 1985) and thus function to maintain the electrochemical gradients across the membrane (Palmgren and Harper, 1999). The activity of proton pumps could influence salt tolerance through affecting the membrane potential and proton gradient. Both factors can affect uptake, exclusion and sequestration of Na^+ and other ions, which are found to have a great impact in salt tolerance. Sequestrating toxic ions in the vacuole or transporting them from the cytosol to the free space against electrochemical potential gradients uses the H^+-gradient as an energy source (Sze, 1985; Serrano, 1985; DuPont, 1992; Garbarino and DuPont, 1988; Palmgren and Harper, 1999). Halophytes use Na^+ and Cl^- as cheap osmolytes sequestering them into the vacuole (Greenway and Munns, 1980).

The plant plasma membrane constitutes the functional interface between cell interior and external environment and is directly involved in the interaction between cytoskeleton and cell wall in the transport of solutes and in the many responses to abiotic and biotic stimuli (Rouquie et al., 1997). Many transport processes involved in growth, plant movement and adaptation to alternating and extreme environmental conditions. It has been proposed that the major deleterious effects of high salinity are caused by Na^+ accumulation in the cytoplasm (Brady et al., 1984; Gibson et al., 1987; Flowers and Yeo, 1988). Plant cells must maintain low cytoplasmic Na^+ if they are going to be tolerant of salt stress (Kiegle and Bisson, 1996). To prevent the accumulation of Na^+, plant may limit its uptake, sequester it in a vacuole, or remove it via the plasma membrane. Aquatic plants are able to use the efflux of Na^+ through the plasma membrane to minimize the irreversible accumulation of Na^+ in the cytoplasm, since this efflux results in the removal of the ions to the aqueous medium surrounding the plant (Kiegle and Bisson, 1996). Control of ion movement across the tonoplast and plasma membranes in order to maintain a low Na^+ concentration in the cytoplasm is the key cellular factor in salinity tolerance (Wu and Seliskar, 1998). When plants are exposed to salinity, the ions, typically Na^+ and Cl^-, lower the external water potential, resulting in turgor reduction or loss, and accumulate excessively in the cytoplasm, leading to inhibition of plant growth and development (Greenway and Munns, 1980). Osmotic adjustment must occur without undue concentration in the cytoplasm of the ions from the saline environment. This is apparently achieved by mechanisms that regulate K^+/ Na^+ selectivity and Cl^- uptake across the plasma

membrane and comparmentalize Na^+ and Cl^- in the vacuole (Greenway and Munns, 1980; Flowers et al., 1977; Jeschke, 1984; Binzel, 1988).

1.8.1 Plasma memabrane H^+-ATPase

Although Na^+/H^+-transporters are responsible for salt tolerance, is unknown whether these proteins are key elements of halophytic plants. However, concentrations above which are in the range of sea water are mostly toxic. Some plants can tolerate enhanced Na^+ concentrations with different strategies. Several genes have been identified for enhancing salt tolerance (*SOS1*) that came from *Arabidopsis thaliana* by an complementation approach with yeast mutants (Qui et al., 2002). Function of *SOS1* includes Na^+/H^+ antiport activity and Na-homeostasis. However, *Arabidopsis* is not typically established in a salt rich environment and it is unknown whether halophytes use similar genes and traits for adaptation. One of the best-characterized plasma membrane marker-enzymes is the H^+-ATPase. In plants, the PM H^+-ATPase acts as the primary transporter that pumps protons out of the cell, thus creating a pH and electrical potential gradient across the plasma membrane that in turn activates many secondary transporters involved in ion and metabolite uptake (Serrano, 1989; Sussman, 1994; Palmgren, 2001). This enzyme was existent in almost all plant cells and allows the identification of this pump by standard techniques such as ATP assays. The regulation of ions across the plasma membrane is thought to be achieved by an electrochemical gradient generated by plasma membrane H^+-ATPase (Sze, 1985). Plasma membrane H^+-ATPase is affected by NaCl as reported in salt tolerant yeast *Zygosaccharomyces rouxii* 5 min after the start of salt stress (Nishi and Yagi, 1992), The plant plasma membrane H^+-ATPase links ATP hydrolysis to the extrusion of protons from the cytoplasm to cell exterior (Briskin, 1990; Michelet and Boutry, 1995). Furthermore, the plasma membrane H^+-ATPase has a major role in a number of important physiological processes, including cell elongation (Rayle and Cleland, 1992), stomatal movements (Assmann, 1993), and cellular responses to a number of factors, including plant growth regulators, light, and fungal toxins (Briskin, 1990; Palmgren, 1991; Briskin and Hanson, 1992).

1.8.2 Vacuolar H^+-ATPase

Salt tolerance of plant cells is mainly achieved by a combination of three different mechanisms: (i) osmotic adjustment of the cytoplasm due to the accumulation of compatible solutes, such as betaine, proline or sugar alcohol; (ii) salt extrusion from the cell across the plasma membrane using ion transporters, such as Na^+/H^+ antiporter (DuPont, 1992; Allen et

al., 1995; Shi et al., 2000); (iii) salt accumulation in vacuoles using tonoplast transporters, such as the Na^+/H^+ antiporter or the Cl^- channel (Matoh et al., 1989; Gaxiola et al., 1999). Vacuoles are the largest organelles of differentiated plant cells occupying up to 90% of the total cell volume (Homeyer, 1989). V-ATPase is emerging as a pump with diverse and surprising functions in eucaryotes. A major role of this pump is to acidify the vacuole, provide energy for transport of ions and metabolites, and so influence turgor and cell expansion (Sze et al., 2002). The accumulation ions in the vacuole serves not only to avoid an increase in the ionic strength of the cytoplasm, but also to increase cellular osmolarity to counter osmotic stress. This latter mechanism is apparent in halotolerant plants, where the succulence of leaf cells is generated by ion accumulation (Mimura et al., 2003). Vacuolar Na^+/H^+ antiport activity was shown first in tonoplast vesicles from red beet storage tissue (Gaxiola, 1999) and later in various halophytic and salt-tolerant glycophytic species (Nass and Rao, 1998; Li et al., 1998). The plant V-type H^+-ATPase (V-ATPase) does not only serve basic housekeeping functions but is also involved in stress-induced NaCl sequestration during salinity stress. By creating the proton motive force across the tonoplast and other endomembranes, like Golgi vesicles, the V-ATPase of higher plants serves, in conjunction with vacuolar H^+-PPase, vital housekeeping functions for cellular metabolism, growth and ion homeostasis (Barkla and Pantoja, 1996; Rea and Sanders 1987; Sze et al., 1992), including sequestration of Ca^{2+} (Chanson 1993; Gelli and Blumwald 1993) and reversible storage of nitrate (Miller and Smith, 1996; Zhen et al., 1991). A recent breakthrough was obtained using transgenic tomato plants which were able to grow, flower, and produce fruits in the presence of 200 mM Na^+ (Zhang and Blumwald, 2001) due to the compartmentation of toxix Na^+ ions in the vacuole. Plant cells are structurally well suited for the sequestration of ions because of the presence of large, membrane-bound vacuoles.

1.9 Changes in the transcriptome of *Aster tripolium* during salt stress

Life depends on the ability of cells to store, retrieve, and translate the genetic instructions required to make and maintain a living organism. DNA stores the information for protein synthesis and RNA carries out the instructions encoded in DNA. The era of microarray hybridization technology began with the simultaneous quantitative determination of mRNA concentrations of a small set of *Arabidopsis* genes by a cDNA microarray (Schena et al., 1995). The cDNA microarray is the most powerful tool for studying gene expression in many different organisms. It has been successfully applied to the analysis of the simultaneous expression of many thousands of genes and to large-scale gene discovery, as well as

polymorphism screening and mapping of genomic DNA clones. It is a high throughput, highly parallel RNA expression assay technique that permits quantitative analysis of RNAs transcribed from both known and unknown genes. There are several reasons why qualitative and quantitative determination of transcript patterns in plant cells is of importance to plant molecular biology. By comparing the concentrations of individual mRNAs present in samples originating from different genotypes, developmental stages or growth conditions, genes can be identified that are differentially expressed and hence may have specific metabolic or morphogenetic functions (Kuhn, 2001). Analysis of transcript patterns should be valuable in assessing roles of novel sequences in an organism, since the similarity of expression patterns of sequences of unknown function with those of known genes may indicate functional homology. Understanding a plant's response to stress will require a comprehensive evaluation of stress induced changes in gene expression. In order to understand salt stress tolerance, functions must be determined for the many stress-regulated transcripts and genes that have not yet been characterized and studied (Bohnert et al., 2001). Until now, the use of microarrays has been limited to a small subset of well-characterized plant species, such as *Arabidopsis*, maize, and rice (Girke et al., 2000; Kawasaki et al., 2001; McGonigle et al., 2000). The small number of suitable organisms places a limit on the biological processes that can be studied. Recently, there was a report of 23 to 47% of the genes on the array was detected, demonstrating that a large number of genes from distantly related species can be surveyed on *Arabidopsis* arrays (Horvath et al., 2003).

1.10 Objectives of the project

Breeding for drought and salinity stress tolerance in crop plants (for food supply) and in forest trees (a central component of the global ecosystem) should be given high research priority in plant biotechnology programs (Wang et al., 2003). Cell biology and molecular genetics research is providing new insight into the plant response to salinity and is identifying genetic determinants that affect salt tolerance. The basic resources for biotechnology are genetic determinants of salt tolerance and yield stability (Yokoi et al., 2002). Implementation of biotechnology strategies to achieve this goal requires that substantial research effort be focused to on identify salt tolerance effectors and the regulatory components that control these during the stress episode (Hasegawa et al., 2000). Plants as sessile organisms are constantly exposed to changes in environmental conditions. When these changes are rapid and extreme, plants generally react with some form of stress response (Aarts and Fiers, 2003).

The aim of this project is to use probable vegetable halophytes *Aster tripolium* L. (already locally cultivated in some Northern parts of Europe) and *Sesuvium portulacastrum* L.

(used locally in South Asia and in tropical coastal area) to understand and elucidate underlying salt stress and tolerance mechanisms. The following different experiments were initiated to study the effect of salt on water stress, gas exchange, ion deficiency and ion toxicity in *Aster* and *Sesuvium*.

(1) Effect of NaCl on uptake of sodium and potassium and their preference over each other during salt stress in *Aster* and *Sesuvium*

(2) Chlorophyll fluorescence and changes in pigmentation (chlorophyll and carotenoid) as a tool for screening salt stress in halophytes *Aster* and *Sesuvium*

(3) Direct effect of NaCl on leaf exchange parameters, organic acids and osmolyte accumulation in *Aster* and *Sesuvium*

(4) Putative role of sulfolipids (SQDG) during salt stress in halophytes and glycophytes

(5) Changes in the enzymatic activity of β-cyanoalanine synthase and cysteine synthase during salt stress in *Aster tripolium*

(6) Finger print analysis of plasma membrane proteins during salt stress in *Aster*

(7) Effect of NaCl on the activities of P-type, V-type and F-type ATPases during salt stress in *Aster* and *Sesuvium*

(8) Analysis of transcriptome changes in *Aster* during salt stress using microarray technology

2 Material and Methods

All medium, buffer, stocks/and working solutions were prepared using double-distilled water.

2.1 Chemicals und other Products

- **Amersham Pharmacia** (Freiburg): [^{33}P] CTP, ATP, Sephadex-G-50 chromatography Nick columns
- **Applichem** (Darmstadt): D-Sorbitol, Dodecyl sulfate sodium salt (SDS), Hydrochloric acid, Magnesium chloride, Sodium chloride, Tris-(hydroxymethyl)-aminomethan (Tris).
- **Carl Roth GmbH CO** (Karlsruhe): Acetone, Acetonitrile, Albumin bovine, fraction V (BSA), 5-Brom-4-chlor-3-indolylphosphat Dinatriumsalt (BCIP), Ethanol, N-2-Hydroxyethylpiperazin-N'-2-ethansulfonicacid (HEPES), Magnesium sulfate, (N-Morpholino)-propansulfonicacid (MOPS), Ninhydrin, p-Nitrotetrazoliumbluechloride (NBT), Salmon sperm DNA, Sodium biocrbonate, Sodium phosphate.
- **Fluka** (Ulm): 2-Butanol, N,N-Dimethyl-p-phenylenediamine dihydrochloride (DMPD), Iodine, Iodoacetamide, Iron(III) chloride, Nitric acid.
- **Gibco BRL** (Karlsruhe): SuperScript II reverse transcriptase
- **Heirler** (Radolfzell): Milk powder
- **J.T. Baker** (B.-V.-Deventer, NL): Disodium hydrogen phosphate, Methanol, Sucrose, Urea.
- **Koch** (Colnbrook, England): Toluol.
- **Merck** (Darmstadt): Ammonium molybdate, Borontrifluoride-methanol complex, Bromophenol blue, Calcium chloride, Cacium sulfate, beta- cyno-4 hydroxycinnamic acid, L-Cysteine, Ethylenedinitrilotetraacetic acid (EDTA), 2-Mercaptoethanol, Perchloric acid, Potassium chloride, Potassium cyanide, Potassium hydroxide, Sodium nitrite, Sodium sulfite.
- **New England Biolabs** (Beverly, USA): T4 polynucleotide kinase
- **Qiagen** (Hilden): Rneasy plant kit
- **Riedel-de-Häen** (Seelze): Potassium nitrate, Potassiumdihydrogen phosphate, Potassiumhydrogen phosphate, Sodium hydroxide.
- **Roche** (Mannheim): CDP-star kit
- **Schleicher & Schuell** (Dassel): Nitran Plus positively loaded Polyamidemembrane
- **Serva** (Heidelberg): Ethidium bromide, Trypsin.
- **Sigma** (St. Louis, USA): O-acetyl-L-serine, Acridine orange zinc chloride double salt (Acridine orange), Adenosine 5'-triphosphate dipotassium salt (ATP), Ammonium carbonate, 1,3 Bis[tris(hydroxy- methyl) methylamino]propane (BTP), 1,4-Dithiothreitol (DTT), Tricine.
- **Koch** (Colnbrook, England): Toluol.

2.2 List of devices

Devices used in the experiments described during the practical work for this thesis was listed below in Table 1.

Apparatus	**Manufacturer**
Autoclave	Varioklav H+P
Casting chamber for SDS-Polyacryl-amide and Tricine-SDS gels	Casting chamber HIS/S. Franziskus PROTEAN II xi Cell/BioRad
Centrifuges	Juoan; RC-5B Refrigerated, Beckman J2-MC, Heraeus SEPATECH megafuge 1.0R, Beckman L8-70M, Beckman Optima TL ultracentrifuge
Electrophoresis apparatus	Mincell EC 370M Electrophoretic Gel System
Incubator	Memmert
Lightings for the climatic chamber	TLD 58W/33/ Philips
Magnetic stirrer with heating plate	MR 3001 K/ Heidolph
PCR-device	PCR-Cycler/ peQlab
pH-Meter	Maxi-M1 Janke&Kunkel/ IKA Werk
Photometer	UVICONXS/ Bio-Tek Instruments
Power supply	Höfer, Biometra-Mini-PP, 2301 Marcodrivel/ LKB Bromma, POWERPAC 1000/ BioRad
Shaker	Certomat R./B. Braun
Thermoblock	Digi-Block JR / Laboratory Devices Inc., USA
Vortexing device	MS2, Minishaker IKA Werk
Waterbath	GFL 1092 and 1086/ Schütt
Weighing device	L610D/ Sartorius Laboratory; 770/ Kern
Western blot device	Roth

2.3 Biological material

Seeds of *Aster tripolium* (Dollart, Germany), *Sesuvium portulacastrum* (Dakhla, Morocco), *Thellungiella halophila* (Arabidopsis stock centre at the Ohio State University), and *Arabidopsis thaliana* (ecotype Wassilewska) were germinated on substrate TKS1 (Floragard, Germany). *Aster* seeds needed a stratification period of 14 d at 4°C on moistened TKS1. The seedlings were transplanted in TKS2 substrate (Floragard, Germany) into pots after about 30 d in the case of *Aster* and 20 days in the case of *Thellungiella* and *Arabidopsis*. At the beginning of the salt treatments the *Aster* plants were about 40 days old having about 5 leaves, the *Arabidopsis* plants were 30 days old having 8 leaves and the *Thellungiella* plants were 45 days old growing as a rosette with more than 20 leaves. *Sesuvium* plants were propagated by making cuttings. The cuttings developed roots in about 7 days. 15 days after making cuttings the salt treatment began. At this state the plants possessed about 8 leaves. The plants were watered daily with nutrient-supplemented tap water without or containing the respective concentrations of NaCl (Sea salt): for the treatment of *Aster* plants 1.5%/257 mM and 3%/513 mM, for *Sesuvium* plants 2.5%/428 mM, 3%/513 mM and 5%/856 mM, for *Arabidopsis* plants 50, 75, and 100 mM, and for *Thellungiella* plants 100, 300, and 500 mM). Conditions in the greenhouse were a 16 h light/8 h dark rhythm at temperatures of 23°C/21°C. When necessary, additional light was switched on for 16 h per day to obtain a constant quantum fluence rate of 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (sodium vapour lamps, SON-T Agro 400, Philips). The salt treatment experiments were repeated three times independently. For analysis 4 plants were combined at each time point.

2.4 Analytical methods

2.4.1 Chromatography

2.4.1.1 Thin layer chromatography (TLC) of SQDG

For SQDG analysis the leaves were ground with a mortar and pestle to a fine powder in liquid nitrogen. 100 mg of the plant material was suspended in 300 µl of methanol:chloroform:water (65:25:4 v/v), mixed and centrifuged for 10 min at 16,060*g* at 4°C. After centrifugation the lower phase (green) was transferred to a new tube and directly applied to a silica gel 60 TLC plate (20 x 20 cm; 0.25 mm layer thickness) (Merck, Darmstadt, Germany). TLC plates were activated at 120°C for 1 h prior to sample application. After loading the samples as spots (5 mm) or streaks (2 cm long) in the case of fatty acids analysis, the plates were developed in solvent (chloroform:methanol:0.02% $CaCl_2$ in the ratio 60:40:9, v/v) in a sealed glass TLC chamber (20 x 20 x 5 cm, Degussa, Germany) at ambient

temperature for about 75 min (modified after Murakami-Murofushi et al., 1985; Archer et al., 1997). For quantification a standard row using commercially available SQDG (Lipid Products, Redhill, Great Britain) of 5 µg/µl diluted to 1:20 for quantification from plant lipid extracts was prepared and applied onto the same plate. Plates were dried for 30 min and then placed into a sealed glass chamber equilibrated before with iodine vapour formed by sublimation (Benning and Somerville, 1992). Spots appeared after about 5 min having. Plates were immediately density scanned because they loose their intensity due to the influence of light. The intensity of the spots was calculated using the TINA 2.0 software package. The determination of SQDG was done two times for each experiment.

2.4.1.2 Fatty acid analysis of SQDG by GC-MS

The SQDG containing fraction was scratched out from the TLC plate and the SQDG was desorbed with 30 ml of methanol under sonification. After concentration *in vacuo*, SQDG was saponified by adding 0.5 ml of 0.5 M methanolic sodium hydroxide and heating to 80°C for 5 min in a tightly screwed derivatisation vial with Teflon septum. For esterification, 1 ml of boron trifluoride methanol complex was added and the mixture was re-heated for 5 min (80°C). After cooling and addition of 2 ml saturated sodium chloride solution, the fatty acid methyl esters (FAME) were extracted with 2 ml of n-hexane.

A Fisons 8060 gas chromatograph equipped with an on-column injector (100°C) was directly coupled with a Fisons MD 800 mass spectrometer. Separation of FAME was achieved on a DB-WAX fused silica column (30 m × 0.32 mm i.d., film thickness 0.25 µm, J & W, Germany) using helium as carrier gas (3.2 ml min^{-1}). The temperature program was as follows: 3 min isothermal at 100°C, raised to 180°C (1 min) at 5°C/min, to 200°C (1 min) at 1°C/min, and to 230°C (5 min) at 5°C/min. The temperature of the ion source was 200°C, the electron energy for the electron impact mass spectra was 70 eV. Assignment of the fatty acids was performed by comparison to the retention indices of authentic reference substances and on the basis of the respective mass spectra.

2.4.1.3 Liquid chromatography - Mass spectrometry of SQDG

Sample application was performed by loop injection (200 µl min^{-1}, CH_3CN/H_2O 1:1 v/v) from a Waters Alliance (Milford, Massachusetts, USA) HPLC system. Mass spectra were recorded in the negative electro spray mode (cone voltage 30 V) by an LCT time of flight mass analyser (Micromass, Manchester, UK), equipped with a LockSpray™ unit for exact mass measurement.

2.4.2 Physiology

2.4.2.1 Determination of chlorophyll

Chlorophyll and carotenoid contents were estimated according to (Lichtenthaler, 1987). 400 µl of ice-cold 80% acetone was added to 50 mg leaf material ground in liquid N_2. The extract was kept on ice for 10 min with mixing every 2 min, centrifuged at 16,060*g* for 5 min at 4°C, and the supernatant was collected for further analysis. The pellet was re-extracted several times until the plant material turned colourless and the supernatant pooled to the supernatant from the first centrifugation step. Finally the chlorophyll content was measured using a spectrophotometer (Uvikon XS, Biotech instruments, Germany) at 646.8, 663.2, and 750 nm and 470 nm for carotenoids. The chlorophyll and carotenoid contents were calculated according to Lichtenthaler (1987). The determination of chlorophyll was done two times for each experiment.

2.4.2.2 Determination of conductivity

The conductivity was determined in soil samples watered with different concentrations of NaCl solutions containing plants or without plants. The soil was completely dried and diluted in the ratio 1 to 250 with tap water (1 g soil/249 ml water). The conductivity was measured using the conductivity meter HI 9033 (Hanna Instruments, Portugal). The instrument was calibrated with standard solutions supplied by the manufacturer.

2.4.2.3 Determination of the osmolarity

Liquid nitrogen frozen leaf material was ground with a pestle and mortar to a fine powder. 350 mg of the powdered leaf was filled in a microcentrifuge tube followed by spinning at 13,000*g* for 20 min. After centrifugation, supernatant cell sap was collected and measured immediately or frozen at –20°C until further use. The osmotic potential was determined cryoscopically by measuring the differing freezing point with a half micro osmometer (Knauer, Berlin, Germany). A 400 mOsmol NaCl solution (12.6872 g H_2O kg^{-1}) was used as a standard.

2.4.2.4 Preparation of enriched plasma membranes (PM) from *Aster tripolium*

PM from *A. tripolium* were enriched according to Larsson et al. (1987) using the phase-partitioning technique with the following modifications: The microsomal fraction was resuspended in a buffer containing 250 mM sucrose, 2 mM KCl, 0.25 mM dithiotreitol

(DTT), 1 mM EDTA, 5 mM K_2HPO_4/KH_2PO_4 (pH 7.8). The upper (U_3) phase was diluted by four volumes of 250 mM sucrose, 1 mM ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), 1 mM DTT, 50 mM 2-amino-2-hxdroxymethyl-1,3-propanediol (Tris)-HCl (pH 7.5) and sedimented at 120,000*g* for 30 min. After washing the PM vesicles were resuspended in 250 mM sucrose, 50 mM KCl, 1 DTT, 3 mM N-(2-hydroxyethyl)piperazine-N´-(2-ethanesulfonic acid)-bis-Tris propane (Hepes-BTP) (pH 7.0), frozen in liquid nitrogen, and stored at -70°C until use.

2.4.2.5 Scanning electron microscope investigations

For scanning electron microscope (SEM) analysis, freshly harvested leaf segments together with droplets of standard solutions were mounted on gold plated specimen holders to avoid corrosion of copper by salts and to prevent background while measuring copper values, and quench frozen with nitrogen slush. The frozen leaf slices taken from the third node were fractured transversely, transferred, and investigated on the cold stage of an ETEC-autoscan SEM. Accelerating voltage was 10 kV and the beam current was 150 µA (Werner and Stelzer, 1990).

2.4.2.6 Energy dispersive X-ray microanalysis and atomic absorption spectrometry

Energy dispersive X-ray-microanalytic analyses (EDXA) were done on bulk-frozen, hydrated and cryocut leaf samples using an ETEC-autoscan scanning electron microscope equipped with a cryostage and a KEVEX/TRACOR-NORTHERN X-ray analysing system. Quantification was carried out by subtracting background from total count harvest and comparing the remaining net counts with those of aqueous standards of 1 M KCl, 300 mM NaCl, 10 mM $CaSO_4$, and 600 mM $MgSO_4$. Element specific X-rays from analysed areas (1 μm^2) within cell vacuoles were registered by a KEVEX Si/Li X-ray detector and computerized by a TN 2000 multi channel analyser. Comparisons between peak to background ratios of specimens and standards were used for quantitative evaluations of the X-ray spectra (Werner and Stelzer, 1990). For atomic absorption spectrometry (AAS) the leaves were washed for 1 min in running ice cooled distilled water. Ion concentrations were analysed in leaf segments of *Aster* and *Sesuvium* divided into 5 or 3 segments starting from the base of the leaf as segment 1 and to the top (segment 5). The tissues were weighed and dried at 65°C overnight. Ions were extracted with 0.5% nitric acid (90°C, 2 h). Na^+ and K^+ were determined directly from the extract by AAS (Type PU 9100x, Philips, Cambridge, Great Britain) at the following wavelength: Na^+ λ = 598.0 nm and K^+ λ = 766.5 nm. Calculations were made using

the direct reading from the AAS to the standards of Na^+ and K^+ (modified after Werner and Stelzer, 1990).

2.4.2.7 Determination of photosynthetic rate, stomatal conductance and leaf gas exchange

Photosynthetic rate, stomatal conductance and gas exchange of a leaf were measured using an LCi portable photosynthesis system (LCi, ADC BioScientific, Herts, Great Britain) in the greenhouse. The instrument is composed of a main console with signal conditioning, air supply, microprocessor control, PC card data storage, keypad, and a leaf chamber connected by a cord. The incoming gas as reference level and the outgoing gas as analysis level build the basis of the measurements. The calculation of the assimilation and transpiration rate is based on the difference of the gas concentration and the airflow rate (Sandra, 2002). Measurement conditions were as follows: water vapour pressure difference (VPD), 13 mbar; photosynthetic flux density (PPFD), 3.500 µmol m^{-2} s^{-1}; CO_2 concentration, ambient CO_2 (C_a 435 µl l^{-1}). Parameters derived from the measurement are stomatal conductance g_s in mol H_2O m^{-2} s^{-1}, transpiration rate (E) in mol H_2O m^{-2} s^{-1}, CO_2-saturated photosynthetic assimilation rate (A) in µmol CO_2 m^{-2} s^{-1}, water use efficiency A/E, and leaf CO_2 concentration C_i in µmol CO_2.

2.4.2.8 Chlorophyll fluorescence measurements, chlorophyll, and carotenoids content

Modulated Chlorophyll fluorescence measurements were made on attached leaves of the plants grown in the climatic chamber (21±2°C, 60% humidity, 12 h light/12 h dark period) with a Mini-PAM portable fluorometer (Walz, Effeltrich, Germany) connected to a notebook computer with data acquisition software (WinControl 1.93). A leaf-clip holder (Model 2030-B, Walz, Germany) was used to hold the leaves. The experimental protocol of Demmig-Adams et al. (1996) was taken as the base model. The minimal fluorescence level (F_o) in dark-adapted state was measured by using modulated light which was too low (<0.1 µmol m^{-2} s^{-1}) to induce any significant variable fluorescence. The maximal fluorescence level in the dark-adapted state (F_m) and the maximal fluorescence level (Fm') during the application of actinic light were measured by a 0.8-s saturating pulse at 8,000 µmol m^{-2} s^{-1}. Actinic light was provided by KL-1500 lamps (Schott, Stafford, UK). The irradiance on the leaf surface was adjusted to a growth light of approximately 50, 310 or 1250 µmol m^{-2} s^{-1}, respectively, for low light, high light or light stress. F_m was measured after 15-20 min of dark adaptation. F_m' was measured at midday with actinic light. The steady-state fluorescence level during

exposure to actinic light (F_s) was also measured. All measurements of the fluorescence were performed with the measuring beam set to a frequency of 600 Hz, whereas all measurements of F_m and F_m' were performed with the measuring beam automatically switching to 20 kHz during the saturating flash. Light intensity was measured by a microquantum-sensor which is measured against a calibrated external quantum sensor (LI-250, LI-1905A, LiCOR, Nebraska, USA) mounted in the leaf-clip holder (Model 2030-B, Walz, Germany). The photosynthetic yield i.e. the actual PSII efficiency (Φ_{PSII}) was calculated according to Genty et al. (1989). The nomenclature used here was according to van Kooten and Snel (1990). Non-photochemical quenching (NPQ) was calculated as (F_m/F_m')-1 according to Bilger and Björkman (1990). Photosynthetic irradiance curves, the so called "rapid light curves", were recorded according to White and Critchley (1999) using 9 times successively increased actinic illumination at an interval of 10 s after which saturation pulse was given every time. Relative electron transport rate (rETR) was calculated following the formula: $rETR = \Phi_{PSII} * PPFD * 0.5 * 0.84$, whereas PPFD refers to photosynthetic active radiation in $\mu mol\ m^{-2}\ s^{-1}$, 0.5 accounts for an equal distribution between the two photosystems, and 0.84 was an average of absorbance of a leaf. Measured values (eg. max. rETR) were analysed and fitted using the model described by Eilers and Peeters (1988).

2.4.3 Enzyme assays

2.4.3.1 Determination of OAS-TL and CAS activities

Enzyme activities were measured with crude protein extracts. To prepare crude extracts plant material was mortared in liquid nitrogen to a fine powder and then added into ice-cold 20 mM Tris-HCl, pH 8.0, in a ratio of 1:10 (100 mg plant material plus 900 µl buffer). The mixture was further homogenized, and the supernatant was adjusted to 1 µg protein per µl for further analysis.

The OAS-TL assay contained in a total volume of 1 ml: 5 mM *O*-acetyl-L-serine, 5 mM Na_2S, 33.4 mM dithiothreitol, 100 mM Tris-HCl, pH 7.5, and enzyme extract (Schmidt 1990). The solutions of *O*-acetyl-L-serine, Na_2S and dithiothreitol were prepared freshly directly before beginning the experiment. The reaction was initiated by the addition of Na_2S and incubated for 30 min at 37°C after which the reaction was terminated by adding 1 ml acidic ninhydrin reagent (0.8% ninhydrin (w/v) in 1:4 conc. HCl:HOAc) to determine the cysteine concentration (Gaitonde, 1967). The samples were heated at 100°C for 10 min to allow colour development and cooled on ice. Finally 2 ml EtOH were added to stabilize the colour complex. The absorbance of the samples was measured at 560 nm. Solutions with different

concentrations of L-cysteine were prepared, treated in the same way as the assay samples and were used for the quantification of the enzymatically formed cysteine. The linearity of the product formation with respect to incubation time and the amounts of protein put into the single assay was carefully tested for each reaction in a similar way as described before (Diessner and Schmidt, 1981).

CAS activity was measured by the release of sulfide from cysteine in the presence of KCN (modified after Blumenthal et al., 1968). The assay contained in a total volume of 1 ml: 0.8 mM L-cysteine, 10 mM KCN, 100 mM Tris-HCl, and enzyme extract. After 15 min at 30°C the reaction was terminated by adding 100 µl of 30 mM $FeCl_3$ dissolved in 1.2 N HCl and 100 µl 20 mM N,N-dimethyl-p-phenylenediamine dihydrochloride dissolved in 7.2 N HCl (Siegel, 1965). The formation of methylene blue was determined at 670 nm in a spectrophotometer. Solutions with different concentrations of Na_2S were prepared, treated in the same way as the assay samples and were used for the quantification of the enzymatically formed sulfide.

2.4.3.2 Proton transport across PM-derived vesicles

The ΔpH was monitored with acridine-orange fluorescence as described by Hellmer et al. (2003). Measurements were performed with a UDL200 laser (Laser Technik, Berlin, Germany) using coumarine 102 as laser dye and a MSG801 SD nitrogene laser (Laser Technik, Berlin, Germany) as pump source. Fluorescence of the samples was excited at a wavelength of 493 nm. The emission spectra were dispersed by a MS125 spectrometer (Lot-Oriel-GmbH) and imaged onto an Instaspec IV CCD camera. To monitor H^+-transport the fluorescence intensity was set at 530 nm wavelength. Proton pumping of PM-derived vesicles (10 µg mL^{-1}) was assayed in 1 mL of test buffer containing 250 mM sucrose, 150 mM KCl, 5 mM $MgCl_2$, 0.1% bovine serum albumin (BSA), 1 mM EDTA, 5 mM 3-(N-morpholino)propanesulfonic acid (Mops)-BTP (pH 6.8), 8 µM acridine-orange, 25 mM $MgSO_4$ and 5 mM K-ATP. To differentiate between the activities of different H^+-ATPases specific inhibitors were used: 50 mM KNO_3 for tonoplast V-type ATPase, 200-400 µM Na_3VO_4 for PM P-type ATPase, and 1 mM NaN_3 for mitochondrial and plastidic F-type ATPase. To investigate activity inhibition by NaCl up to 300 mM were added.

2.4.3.3 Measurements of ATPase activities

The preparation of cell homogenates and the determination of ATPase activities were done according to Koyro et al. (1993) with some modifications: *Aster* and *Sesuvium* leaves were

ground in a mortar in the presence of liquid nitrogen. The powdered leaf material was suspended in the following medium (2 g in 10 ml): 25 mM HEPES/KOH, pH 7.4, 600 mM sorbitol, 10 mM $MgCl_2$, and 50 mM KCl. The mixture was filtered though nylon net (pore size 30 µm) and immediately used for the enzyme assays. 50 µl was taken for the chlorophyll estimation. For the ATPase assays 0.2 ml of leaf extract was incubated in a medium containing 25 mM HEPES/KOH, pH 7.4, 600 mM sorbitol, 10 mM $MgCl_2$, 50 mM KCl, 0.5 mM ATP and 2 µl ATP [γ-^{32}P] (10 MBq) (Hartmann Analytik, Braunschweig, Germany). The assay mixture was kept at 37°C in a water bath for 15 min. The reaction was stopped by adding 3 M $HClO_4$ to a final concentration of 0.3 M. After centrifugation for 5 min at 9,000*g*, 0.2 ml of the supernatant was mixed with 1 ml of 1 g ammonium heptamolybdate [$(NH_4)Mo_7O_{24}$ x 4 H_2O] in 100 ml 1 M $HClO_4$, then 50 µl of the molybdate complex was added to 2 ml of a szintillation cocktail (Ready value, Beckman, USA) and the samples were placed into a szintillation counter (LS 1801, Beckman Instruments, Fullerton, California) for total counts of [^{32}P]. To obtain the amount of labelled hydrolysed phosphate (P_i) 1 ml of a mixture of iso-butanol and toluol (1:1, v/v) was added to the remaining molybdate complex and mixed for 20 s. To 0.5 ml of the upper organic phase 2 ml szintillation cocktail were added and placed into the szintillation counter. To differentiate between different ATPases following inhibitors were added to final concentrations of 1 mM V_2O_5 to inhibit P-type ATPases, 40 mM KNO_3 to inhibit V-ATPases or 5 mM NaN_3 to inihibit F-type ATPases. The pH of the inhibitor solutions was adjusted to pH 7.4.

2.5 Proteomics

2.5.1 SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting

For the determination of OAS-TL and CAS steady-state protein levels in the plants 100 mg plant material was mortared to a fine powder in liquid nitrogen. 500 µL sample buffer (56 mM Na_2CO_3, 56 mM dithiothreitol, 2% SDS, 12% sucrose, 2 mM EDTA) was added, samples were incubated for 20 min at 95°C and centrifuged. About 10 µg of the total protein supernatant was subjected to SDS-PAGE (Laemmli, 1970) and stained with Coomassie-Brilliant Blue or blotted (Sambrook et al., 1989). Antibodies directed against purified spinach OAS-TL and purified spinach CAS (Hatzfeld et al., 2000) were used for the immunodetection. A colorimetric detection method using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate was applied. Protein estimation was done according to Bradford (1976) using bovine serum albumin as a standard.

2.5.2 SDS-PAGE and Western blotting of PM proteins

For the separation of PM proteins 10 µg of the PM fraction were solubilized with 6x loading buffer (350 mM Tris-HCl, 10.28% [w/v] sodium dodecyl sulfate (SDS), 36% [v/v] glycerol, 5% 2-mercaptoethanol, 0.012% [w/v] bromphenol blue) and was subjected to denaturing SDS polyacrylamide gel electrophoresis (PAGE) according to Laemmli (1970) and blotted onto a nitrocellulose membrane (Sambrook et al., 1989). After blocking with 5% milk powder the membrane was incubated overnight at 4°C with the 2,000-fold diluted primary antibody, which was raised against the conserved cytoplasmic region (central loop) of the PM H^+-ATPase from *Oryza sativa* (OSA1; Wada et al., 1992). The membrane was incubated for 1 h with a 2,000-fold diluted secondary antibody (goat anti-rabbit immunoglobulin antibody conjugated with alkaline phosphatase). For staining a colorimetric detection method using nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) was applied. Aliquots of 5µl from each preparation were used for protein determination according to Legler et al. (1985).

2.5.3 Two-dimensional gel electrophoresis of PM proteins

The PM proteome was analyzed by isoelectric focusing (IEF) and following N-tris(Hydroxymethyl)methylglycine (Tricine) SDS-PAGE. IEF was carried out with the IPGphor system Amersham Pharmacia Biotech AB (Uppsala, Sweden) using Immobiline DryStrip gels (18 cm) with nonlinear pH gradients (pH 3-10) according to the manufacturer's instructions (Berkelmann and Stenstedt, 1998). Proteins (250 µg) were resuspended in 40 µL of lysis solution buffer (8 M urea, 4% [w/v] 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 40 mM Tris-HCl, pH 8.8, 50 mM DTT), incubated for 1 h at room temperature, and subsequently supplemented with 260 µL of the corresponding DryStrip rehydration solution (8 M urea, 2% [w/v] CHAPS, 0.2% [v/v] of a carrier ampholyte mixture [Bio-Lyte 3/10, Bio-Rad Laboratories, Munich, Germany], a trace of bromphenol blue, and 50 mM DTT). The solution was directly applied onto a dry gel strip; rehydration took place actively for 12 h and focusing at 10,000 V for 7 h. After IEF gel strips were incubated with equilibration buffer A and B for 20 min each (equilibration buffer A: 50 mM, Tris-HCl, pH 8.8, 6 M urea, 30% [v/v] glycerol [87%], 2% [w/v] SDS, 1% [w/v] DTT, and a trace of bromphenol blue, and equilibration buffer B: 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% [v/v] glycerol [87%], 2% [w/v] SDS, 2.5% [w/v] iodoacetamide, and a trace of bromphenol blue). The strips were transferred horizontally onto a Tricine SDS polyacrylamide gel according to the manufacturer's instructions (Bio-Rad). Tricine SDS-PAGE (10%) was carried out in the

Protean II Xi cell from Bio-Rad (gel dimensions 20 × 18 × 0.1 cm) according to the protocol given by Schägger and von Jagow (1987). The proteins were visualized by silver staining (Heukeshoven and Dernick, 1986).

2.5.4 Peptide finger print analysis by mass spectrometry of PM proteins

For mass spectrometry (MS), gels were stained with Coomassie Colloidal Blue (Neuhoff et al., 1990). Single protein spots were cut out and transferred onto a 96 wells microtiter plate for Liquid Handling MassPrep station (Micromass) convenience. The spots were washed 3 times each with acetonitrile and 25 mM ammonium carbonate buffer, pH 8.0. The reduction step was performed with 10 mM DTT and 25 mM ammonium carbonate buffer, pH 8.0, at room temperature for 1 h. The reduction solution was then removed and 55 mM iodoacetamide and 25 mM ammonium carbonate, pH 8.0, were added. The alkylation step was carried out for 30 min at room temperature. After another rinse with the ammonium carbonate buffer and dehydration steps, a small volume of 12 ng μL^{-1} trypsin was added manually according to the spot size, and the plate was incubated at 37°C for at least 8 h. Afterwards, digestion peptides were extracted from the gel with 5 µL of 60% acetonitrile/H_2O (v/v) solution under sonication. Mass measurements were carried out on the BIFLEX III^{TM} Matrix-Assisted Laser Desorption Time-Of-Flight Mass Spectrometer (MALDI-TOF) (Bruker, Bremen, Germany) equipped with the SCOUT^{TM} High Resolution Optics with X-Y multisample probe and griddles reflector. This instrument was used at a maximum accelerating potential of 19 kV and was operated in reflector mode. Ionisation was accomplished with a 337 nm beam from a nitrogen laser with a repetition rate of 3 Hz. The output signal from the detector was digitalized at a sampling rate of 2 GHz. A saturated solution of β-cyano-4-hydroxycinnamic acid in acetone was used as a matrix. A first layer of fine matrix crystals was obtained by spreading and fast evaporation of 0.5 µL of matrix solution. On this fine layer of crystals, a droplet of 0.5 µL aqueous formic acid (5%) solution was deposited. Afterwards 0.5 µL of sample solution was added and a second droplet 0.2 µL of matrix saturated solution (in H_2O/acetonitrile, 1:1) was added. The preparation was dried under vacuum. The sample was washed one to three times by applying 1 µL of aqueous formic acid (5%) solution on the target and then flushed after a few seconds. The calibration was performed in internal mode with trypsin autolysis fragments at *m*/*z* 842.51 and 2211.107. Protein identification has been performed with the Protein Prospector (MS-Fit) and Prowl (Profound) search tools on the NCBI databank within 50 ppm peptide mass tolerance.

2.6 Molecular biological methods

2.6.1 RNA isolation and radiolabelling

Total RNA was extracted from the leaf material ground in liquid N_2 from *Aster tripolium* 0% and 3%/513 mM NaCl treated plants (RNeasy Plant Kit, Qiagen GmbH, Germany). Concentration and quality of isolated RNA was monitored on electropherograms by 2100 Bioanalyser (Agilent Technologies). For reverse transcription and radiolabelling, 10 µg total RNA was used. Radiolabelling was performed as described by Thimm et al. (2001). For reverse transcription and radiolabeling, 10 µg total RNA was used (SuperScript II, GibcoBRL, Karlsruhe, Germany; [^{33}P]CTP, Amersham Pharmacia, Freiburg, Germany). After transcription, RNA was hydrolyzed with NaOH (0.25 N) and neutralized with HCl (0.2 N) and sodium phosphate buffer (40 mM, pH 7.2). Labeling efficiency was controlled by scintillation countering (LS6500, Beckman, Munich) after removal of unincorporated oligonucleotides by Sephadex G-50 chromatography (NICK Columns, Amersham Pharmacia).

2.6.2 Array hybridisation and data analysis

Filter design and production, as well as reference and complex hybridisations were performed as described by Thimm et al. (2001). A set of 16,128 cDNA clones from the Michigan State University (East Lansing) collection, characterized by EST analysis (Newman et al., 1994), was provided by the Arabidopsis Biological Resource Center (Columbus, OH). The cDNA was amplified by PCR using LacZ-specific primers (forward LacZ1 5' GCTTCCGGCTCGTATGTTGTGTG 3' and reverse LacZ2 5' AAAGGGGGATGTGCTGCAAGGCG 3'). The PCR products were spotted automatically onto nylon membranes (Biogrid, Biorobotics, Cambridge, UK; Nytran Supercharge, 22.2 × 22.2 cm, Schleicher and Schüll, Dassel, Germany). PCR products were not checked on an agarose gel for contamination before spotting. To normalize the amount of spotted cDNA, a reference hybridization for each filter was carried out using [^{33}P]-labeled PCR product-specific primer (T4 polynucleotide kinase, New England Biolabs, Beverly, MA; [^{33}P]ATP, Amersham Pharmacia; 5' TTCCCAGTCACGA 3'). The filters were hybridized at 5°C overnight and washed for 40 min at 5°C in SSarc (4× SSC, 7% [v/v] Sarcosyl NL30, and 4 µM EDTA). Filters were exposed for 16 h on imaging plates and detected with a phosphoimager (BAS-1800, Fuji, Tokyo). Radioactivity was removed from filters by washing two times in SSarc at 65°C for 30 min. After prehybridization for 2 h at 65°C in Church buffer (7% [w/w] SDS, 1 mM EDTA, pH 8.0, and 0.5 M sodium phosphate, pH 7.2) containing salmon sperm DNA (100 ng ml^{-1}, Roth, Carl GmbH & Co, Karlsruhe, Germany), filters were

hybridized with the labeled cDNA probe at 65°C for 36 h. Washing steps were carried out at 65°C for 20 min each with 1× SSC, 0.1% (w/v) SDS, 4 mM Na_2PO_4 (pH 7.2); 0.2× SSC, 0.1% (w/v) SDS, 4 mM Na_2PO_4 (pH 7.2); and 0.1× SSC, 0.1% (w/v) SDS, 4 mM Na_2PO_4 (pH 7.2). The filters were exposed on imaging plates for 16 h and signals were detected using a phosphorimager (BAS-1800 II, Fuji) followed by stripping for 1 h at 65°C (0.1% [w/v] SDS and 5 mM Na_2PO_4, pH 7.2) as above. Hybridization of each filter was repeated three times with a newly synthesized and labeled cDNA probe of the corresponding RNA pool.

2.6.3 Data Analysis

For data analysis, the signal intensities of the reference and experimental hybridisations were quantified using the software ARRAYVISION (Imaging Research Inc., Haverhill, UK). Normalisation, statistical analysis and response evaluation were done using the mathematical tools incorporated into the Haruspex database (http://www.mpimp-golm.mpg.de/haruspex/index-e.html), as described by Thimm et al. (2001). In total, eight filters were hybridised up to five times each with newly synthesised and labelled cDNA probe of the corresponding RNA pool. To control the influence of the individual filters to the hybridisation quality, two filters were cross-hybridised with the same RNA pools. Possible functions of ESTs were obtained via clone identification numbers or sequences from online databases (TIGR Arabidopsis Gene Index, Institute for Genomic Research, Rockville, MD; The Arabidopsis Information Resource, Stanford, CA; and BLAST, National Center for Biotechnology Information, Bethesda, MD). Throughout this manuscript, mention of EST clone ID has not been made.

2.6.4 Northern blot analysis

Total RNA was extracted essentially as described (Chomczynski and Sacchi, 1987). RNA samples (25 µg) were separated on 1% denaturing agarose-formaldehyde gels. Equal loading was controlled by staining the gels with ethidium bromide. After RNA-transfer onto nylon membranes (Sambrook et al., 1989), filters were probed with digoxigenin-labeled (Roche, Mannheim, Germany) cDNA probes obtained by PCR. Colorimetric or chemiluminescent detection methods with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) or with CDP-*Star* (Roche, Mannheim, Germany) as substrates for alkaline phosphatase were applied.

3 Two halophytes, two strategies in salt tolerance

3.1 INTRODUCTION

Soil salinity is a major constraint to food production because it limits crop yield and restricts use of land previously uncultivated. To feed the world growing population productivity has to be increased by 20% in the developed world and by 60% in the developing world and so the best possible use must be made of these once productive soils (Owens, 2001). Halophytes are naturally evolved salt-tolerant plants that are adapted to growth in environments that inhibit the growth of most glycophytic crop plants. While studies on glycophytes provide evidence that genetic variability for salt tolerance exists, the actual mechanisms and molecular basis underlying salt tolerance in halophytes are not well understood. The sea aster *Aster tripolium* L. belongs to the Asteraceae family and grows naturally in the upper salt marsh and coastal areas of temperate regions. Since it has no morphological characteristics that would allow to exclude salt, *Aster* is thought to have developed adaptation mechanisms to salt stress at the cellular level. *Sesuvium portulacastrum* L. belongs to the Aizoaceae family and grows naturally in the subtropical, Mediterranean coastal, and warmer areas around the world. Both halophytes are potential crop plants either as vegetables or as animal fodder, respectively. As a creeping plant *Sesuvium* can also be used to avoid soil erosion and beautification in deserted landscapes. Our approach is to differentiate between the strategies of both halophytic species in resisting to short-term salt stress conditions.

Generally, photosynthetic activity is suppressed under salt stress and it is severely suppressed in salt-sensitive species such as in most glycophytic crop plants (Seemann and Critchley, 1985). One can differentiate two points: “stomatal limitation” and “non-stomatal limitation”. Stomatal limitation means that the photosynthetic rate declines due to a reduction in the level of CO_2 supply as a result of stomatal closure. Non-stomatal limitation occurs by a decrease in the activity of CO_2 fixation in the mesophyll cells because of water loss or excessive salt concentration (Ueda et al., 2003). The responsible factors for controlling net CO_2 assimilation in plants are stomatal conductance, carbon uptake, photochemical capacity, or a combination of all these (Lawlor, 2002). Another important strategy of halophytes is associated with osmotic adjustment. High salinity causes hyperosmotic stress and ion disequilibrium that produce secondary effects or pathologies (Hasegawa et al., 2000; Zhu, 2001). Metabolic acclimation via the accumulation of compatible solutes is regarded as a basic strategy for the protection and survival of plants in extreme environments (Sakamoto

and Murata, 2000). The compounds involved in osmotic adjustment differ whether the plant has low salt content in the shoots (excluder) or high salt content (includer) (Daoud et al., 2002). The extent of Na^+ and K^+ accumulation will determine either way of excluder or includer and hence one of the most important mechanism to enable tolerance of high salt.

Changes in the photosynthetic parameters could potentially be used as a screening method for salt tolerance in plants, because more tolerant cultivars are expected to exhibit fewer disturbances in the photosynthetic processes when growing under salinity (Belkhodja et al., 1999). In recent years, the technique of chlorophyll fluorescence, a tool that monitors the function of the photosynthetic apparatus, has become ubiquitous in plant ecophysiology studies. The decrease in photosynthesis induced by salt stress enhances the amount of excess excitation energy (Björkman, 1989), which, if not safely dissipated, may result in photodamage to photosystem II (PSII) because of an over-reduction of reaction centers (Demmig-Adams and Adams, 1992).

Maintaining plant growth in saline conditions depends in part on the ability to keep cytoplasmic Na^+ levels low to protect the Na^+-sensitive metabolic machinery. While cellular metabolism in halophytes is also sensitive to high levels of Na^+, these plants can utilize at least one of the four mechanisms to prevent Na^+ accumulation in the cytoplasm: reducing Na^+ entry into the cell, active Na^+ efflux from the cell, active sequestration of Na^+ in the vacuole and osmotic adjustment in the cytoplasm (Parks et al., 2002). Transport activated by the plant H^+-ATPases is involved in many physiological functions, e.g. mineral nutrition in the root, metabolite translocation, regulation of cytoplasmic pH, and cell turgor-related functions, such as organ movement and cellular growth (reviewed in Briskin and Hanson, 1992; Sussman, 1994; Morsomme and Boutry, 2000; Palmgren, 2001). The proton and electrical gradient produced by the plasma membrane (PM) H^+-ATPase is the driving force for active secondary transport and the regulation of Na^+ and Cl^- uptake (Niu et al. 1995). Vacuolar Na^+ accumulation could be accomplished by the combined activities of H^+ pumps and a Na^+/H^+ exchanger on the vacuolar membranes (Parks et al., 2002). Decreased ATP synthesis by the organellar H^+-driven ATP synthase was considered to be the primary effect of decreasing relative water content and due largely, but not exclusively, to the effects of increasing ion (specifically Mg^+) concentrations in the chloroplast as the relative water content falls (Keck and Boyer, 1974; Younis et al., 1979; Tezara et al., 1999). To understand the strategies of *Aster* and *Sesuvium* it is important to know which of the three ATPases is active during salt stress and hence understanding the process of accumulation or extrusion of ions toxic to the cells. Halophytes such as mangroves have developed morphological and physiological

adaptations to their environment such as salt glands, leaf succulence, and ultrafiltration by roots (Albert, 1982; Tomlinson, 1986). Based on these findings, we are particularly interested to look for ultrastructural tissues in *Aster* for ion compartmentation inside the cell other than vacuoles.

The results of ion concentrations and water relations, photosynthetic parameters including leaf gas exchange, the ratio of chlorophyll a to b, the carotenoid content, photochemical reactions based on chlorophyll fluorescence, bioenergetics during salt stress by analysing P-, V-, and F-type ATPase activities in *Aster* and *Sesuvium* will be described.

3.2 RESULTS

3.2.1 Effect of salt treatment on the phenotype of the *Aster* and *Sesuvium* plants

For the experiments done here the amounts of NaCl added to the watering solutions were determined in pre-tests to obtain similar effects for each plant species with respect to growth retardation and reduction of fresh weight increase (data not shown). In *Aster* and *Sesuvium* the growth was reduced after 10 days of salt treatment with the NaCl concentrations indicated (Fig. 1a and b).

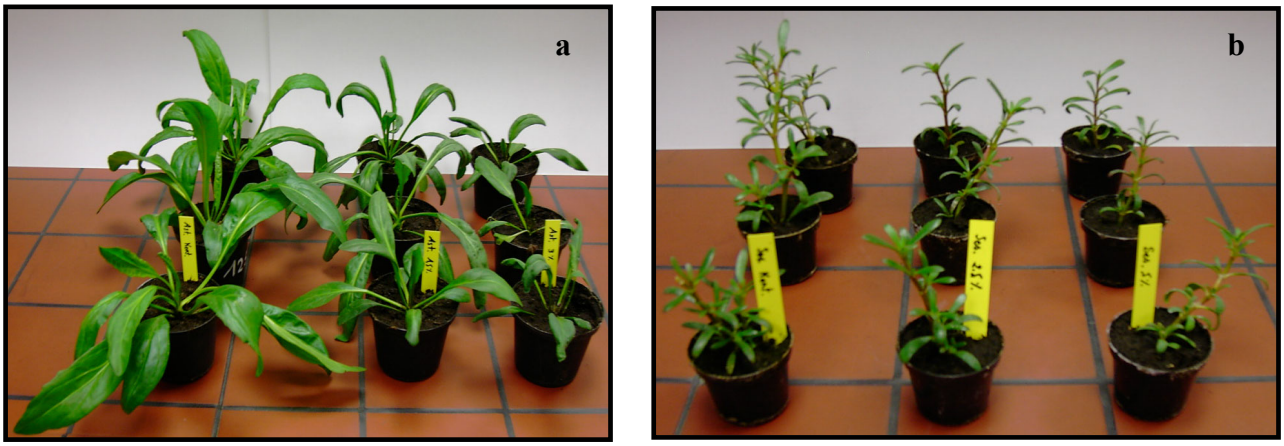


Fig. 1a,b Survivability and growth of *Aster tripolium* L. and *Sesuvium portulacastrum* L. plants cultivated with different salt concentrations in the watering solutions. **a** *Aster* (from the left to the right, 0%, 1.5%/257 mM, and 3%/513 mM NaCl). **b** *Sesuvium* (from the left to the right, 0%, 2.5%/428 mM, and 5%/856 mM NaCl) in the watering solution for up to 10 days. The seedlings were transplanted in TKS2 substrate into pots after about 30 days. Conditions in the greenhouse were a 16 h light/8 h dark rhythm at temperatures of 23°C/21°C, with a humidity around 55%. When necessary, additional light was switched on for 16 h per day to obtain a constant quantum fluence rate of 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The plants showed decreased growth rates with increasing salt concentrations.

Aster grew on NaCl levels between 0 and 3%/513 mM close to seawater salinity of 3.5% (Fig. 1a). The succulent plant *Sesuvium* was more tolerant to NaCl and could grow at NaCl concentrations up to 5%/856 mM with a similar percentage in growth reduction as *Aster*

plants showed at 3%/513 mM (Fig. 1b). In *Aster* the LMA (Leaf mass to area) decreased slightly to 2.05 g dm^{-2} in 1.5%/257 mM watered plants when compared to the control having 2.49 g dm^{-2} whereas in the 3%/513 mM NaCl plants any difference in the LMA could be observed. In *Sesuvium* the LMA decreased at the 5%/856 mM treatment to 3.83 g dm^{-2} from 4.28 g dm^{-2} of the untreated plants. However, the LMA increased to 5.53 g dm^{-2} in 2.5%/428 mM NaCl treated plants compared to the control.

3.2.2 Ion analysis and water relations

Leaf ion contents (Na^+ and K^+) were determined in both Aster and Sesuvium and expressed based on dry weight (Fig. 2a-d). *Aster* leaves the amount of Na^+ increased with increasing NaCl concentrations. When looked upon segmental variation, the higher concentrations were stored at the tip of the leaf tissue (segment 5) and gradually decreased to the base of the leaf (segment 1). In *Sesuvium* also the amount of Na^+ was enhanced with increasing NaCl concentrations, but the concentration of Na^+ accumulation was double as high as in *Aster*. Segmental distribution of Na^+ in the leaf tissue of *Sesuvium* showed no significant upstream or downstream as was observed in *Aster*. The scenario was different with the K^+ concentration in both plants. In *Aster* the accumulation of K^+ was about two fold higher than Na^+, but any changes between the control and NaCl treated plants could be determined. K^+ allocation in the leaf of *Aster* was highest in segment 2 and decreased to segment 5 of the leaf opposite to Na^+ stored at the tip. In *Sesuvium* the reverse was shown with almost 75% less K^+ accumulation than Na^+ in the leaf tissue. The segmental differentiation for K^+ was seen in the middle segment (segment 2) at all NaCl concentrations. The ratio of Na^+ to K^+ in *Aster* was 0.33, 0.52, and 0.65 for 0%, 1.5%/257 mM, and 3%/513 mM, respectively. The Na^+/K^+ quotients for *Sesuvium* plants treated with 0%, 2.5%/428 mM, 3%/513 mM, and 5%/856 mM were 4.2, 6.61, 5.61, and 6.91, respectively. The analysis of ion concentrations in the mesophyll vacuoles of *Aster* using SEM were done to compare the data obtained through AAS which in theory should be comparable. In the vacuole the amount of Na^+ ions increased clearly with increasing NaCl concentrations in the watering medium whereas the K^+ amount is relatively unchanged within control and NaCl treated *Aster* leaf vacuoles (Table 1). The relative water content remained almost constant within all NaCl concentrations in *Aster* and *Sesuvium* ranging from 93-95% and 91-93%, respectively (data not shown). The effects of NaCl in the watering solution became evident by measuring the osmolarity in both *Aster* and *Sesuvium* which increased with increasing NaCl

concentrations (Table 2). On average the osmolarity was about 30% higher in *Sesuvium* compared to *Aster*.

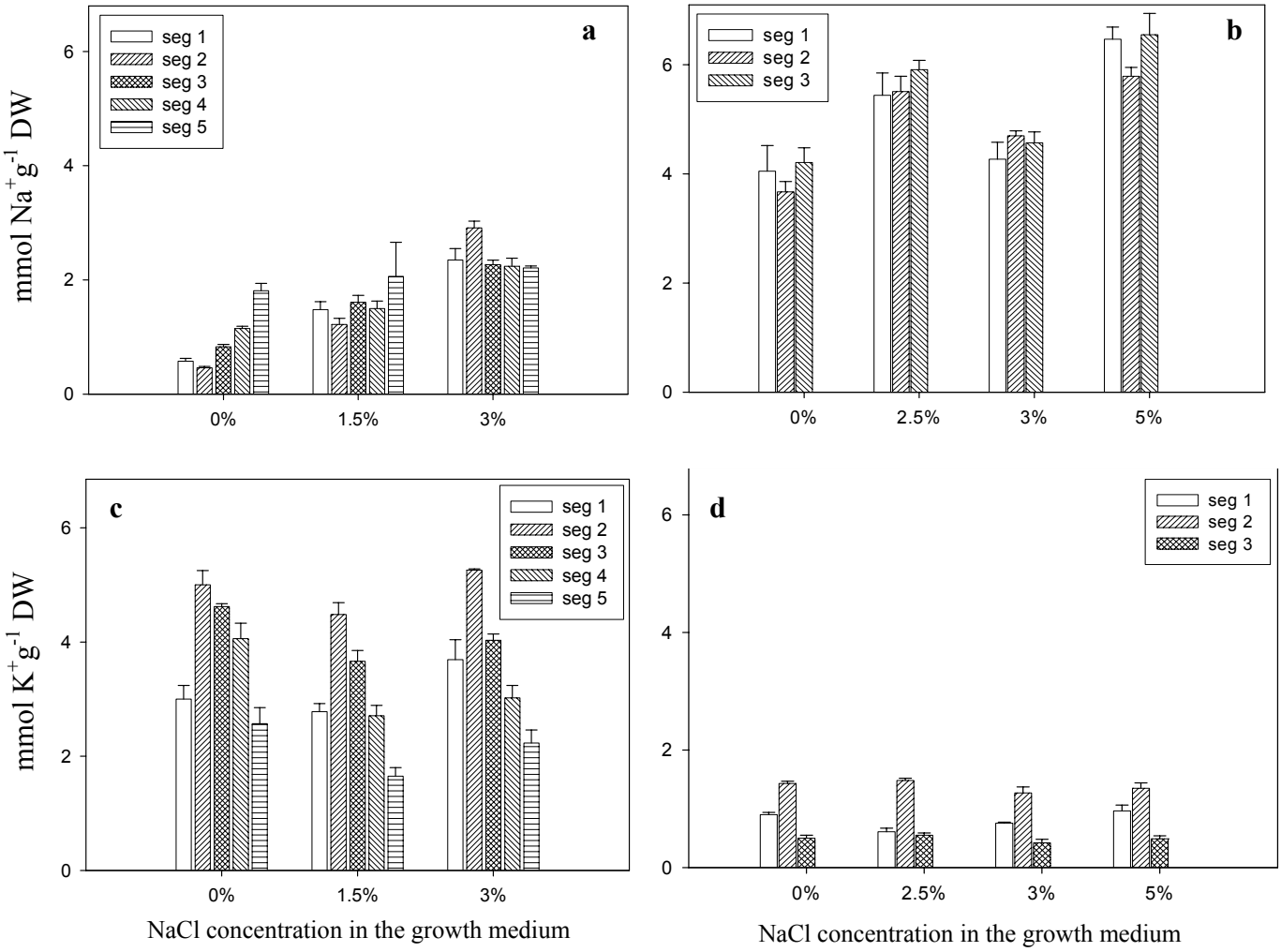


Fig. 2a-d Na^+ and K^+ contents in segments of *Aster* and *Sesuvium* leaves from the control and NaCl treated plants based on tissue water content (0%, 1.5%/257 mM, and 3%/513 mM or 0%, 2.5%/428 mM, 3%/513 mM, and 5%/856 mM NaCl, respectively). The segments were cut from the bottom (segment 1) to the top (segment 5 or 3, respectively). **a** Na^+ concentration in leaf segments of *Aster*. **b** Na^+ concentration in leaf segments of *Sesuvium*. **c** K^+ concentration in leaf segments of *Aster*. **d** K^+ concentration in leaf segments of *Sesuvium*. Leaves were harvested on day 5 of the experiment al period lasting for 10 days. Ions were extracted with 0.5% nitric acid (90°C, 2 h). Na^+ and K^+ were determined directly from the extract by atomic absorption spectrometry (AAS) at the following wavelengths: Na^+ λ = 598.0 nm and K^+ λ = 766.5 nm.

Table 1 Ions (Na^+, K^+, and Cl^-) concentration in the mesophyll vacuole of *Aster* watered with 0%, 1.5%/257 mM, and 3%/513 mM NaCl in the watering solution and analysed on day 5 after beginning of the treatment.

	Concentration of ions in the mesophyll vacuole (mM)		
NaCl conc.	Na^+	K^+	Cl^-
0%	9±3	148±28	16±7
1.5%	149±9	397±80	182±15
3%	305±188	171±6	101±27

Table 2 Osmolarity in *Aster* and *Sesuvium* watered with 0%, 1.5%/257 mM, and 3%/513 mM or 0%, 2.5%/428 mM, and 5%/856 mM NaCl, respectively, in the watering solution and harvested on day 1, 3, 5, and 10 starting one day after beginning of the treatment.

	Osmolarity in *Aster* (mOsmol kg^{-1})			
NaCl conc.	day 1	day 3	day 5	day 10
0%	386±94	386±97	389±98	370±23
1.5%	431±95	475±39	513±11	698±31
3%	443±98	521±31	615±53	923±35
	Osmolarity in *Sesuvium* (mOsmol kg^{-1})			
NaCl conc.	day 1	day 3	day 5	day 10
0%	473±6	458±13	444±1	458±8
2.5%	510±3	624±82	721±149	901±189
5%	507±57	720±66	951±144	1300±81

3.2.3 Photosynthetic characteristics: gas exchange

The CO_2-saturated photosynthetic assimilation rate (A) expressed in µmol CO_2 m^{-2} s^{-1} was measured with varying PPFD (almost 0 to 3,500 µmol m^{-2} s^{-1}) at the beginning of the experiment (day 0) and at day 10 at plants watered with different NaCl concentrations (Fig. 3a and b). *Aster* reached the highest assimilation rate at the maximum PPFD applied, but in *Sesuvium* a beginning saturating activity was observed at about 1,500 µmol m^{-2} s^{-1} PPFD.

Interestingly, the (sub-)tropical plant *Sesuvium* is not as effective at higher light intensities in its assimilation rate as *Aster*.

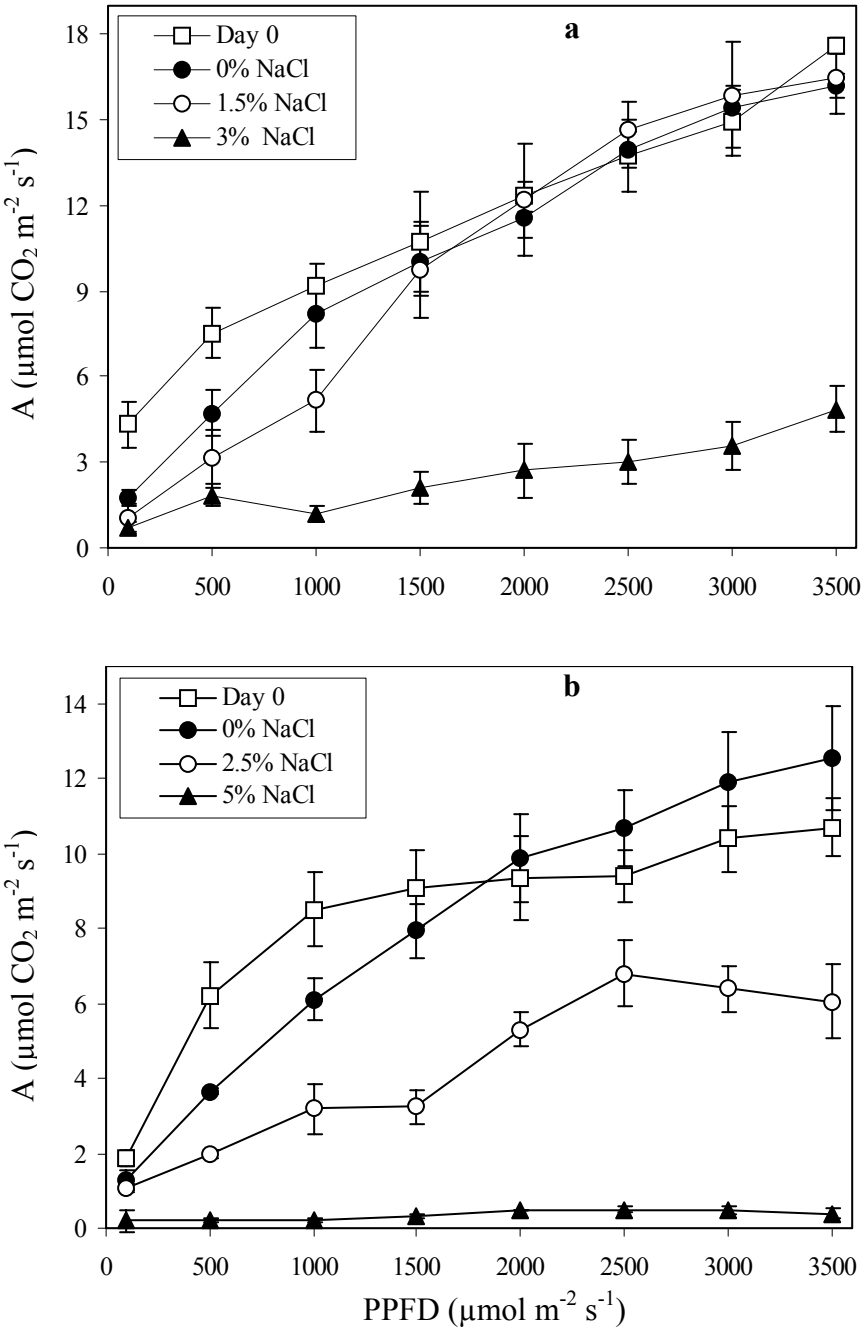


Fig. 3a,b The CO_2-saturated photosynthetic assimilation rate (A, given in µmol CO_2 m^{-2} s^{-1}) was measured in controls and salt-treated *Aster* and *Sesuvium* plants on day 0 and day 10 at different light intensities. The assimilation rate was plotted against the photosynthetic photon flux density (PPFD) given in µmol m^{-2} s^{-1}. **a** Data collected from *Aster*. **b** Data collected from *Sesuvium*. The measurements were made always in the morning at 9-10 am three hours after switching on the additional light. CO_2 assimilation rate (A, given in µmol CO_2 m^{-2} s^{-1}) in the leaves were measured using a LCi portable photosynthesis system in the greenhouse. Measurement conditions were as follows: leaf temperature, 29±2°C; water vapour pressure difference (VPD), 13 mbar; PPFD, from 500 to 3.500 µmol m^{-2} s^{-1}; CO_2 concentration, ambient CO_2 (C_a 435 µl l^{-1}) (Sandra 2002). The measurements were made in the leaves from different plants for each of the NaCl concentrations.

The measurements at day 10 showed that the controls remained as active as at the beginning of the experiment, but both plants species showed drastically reduced assimilation rates at the highest NaCl concentrations applied.

The CO_2-assimilation rate in *Aster* plants watered with 3%/513 mM NaCl was already affected at early time points of the experiment compared with 0% and 1.5%/257 mM NaCl plants (Fig. 4a). Also in *Sesuvium* the effects of reduced assimilation rates could be observed in 5%/856 mM NaCl treated plants already at the beginning of the experiment. However, the decrease in assimilation to almost unmeasurable levels was only shown after 10 days of treatment at the higher salt concentration applied (Fig. 4b).

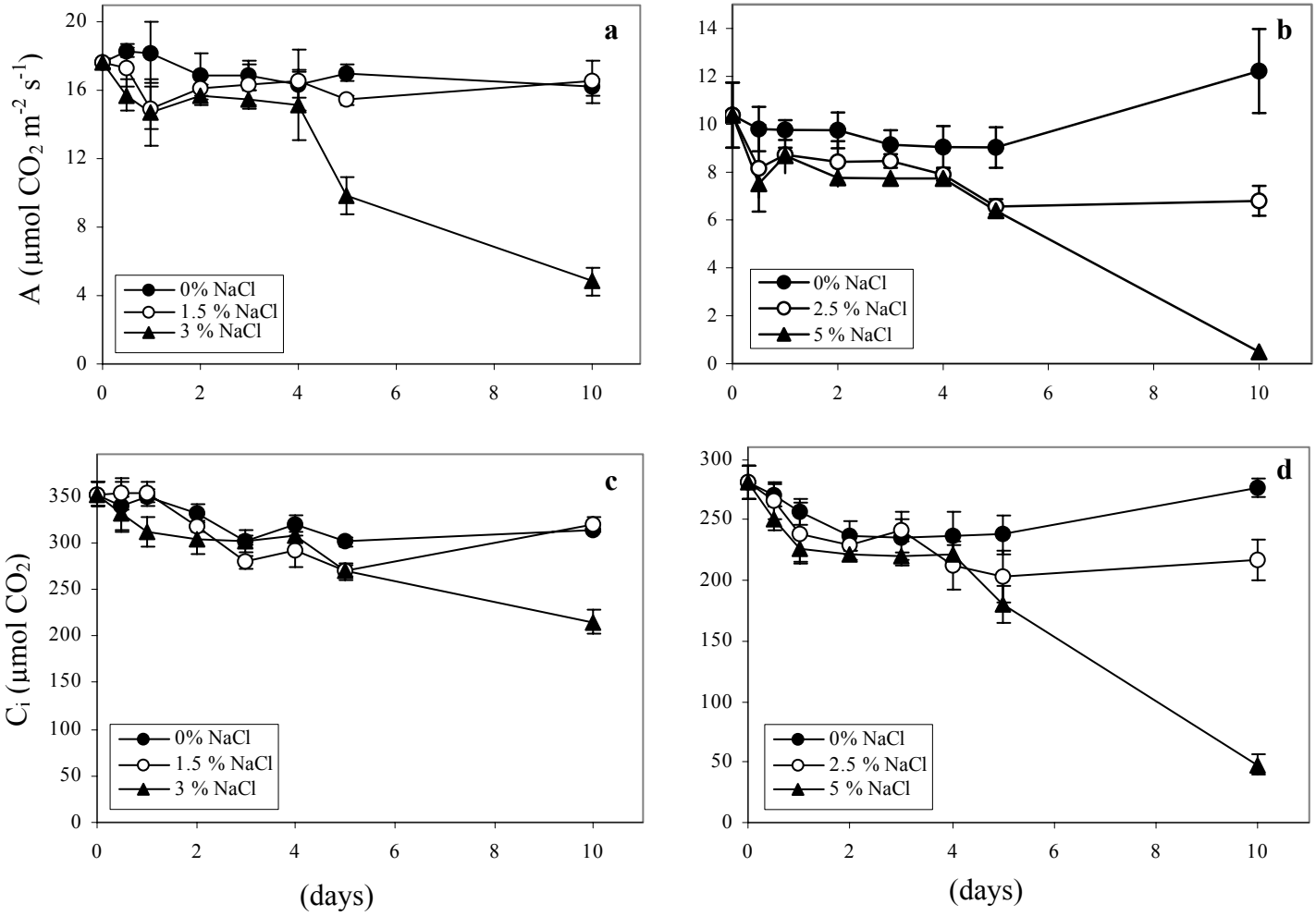


Fig. 4a-d Instantaneous CO_2-saturated photosynthetic assimilation rate (A, given in µmol CO_2 m^{-2} s^{-1}) and in parallel the intercellular leaf CO_2 concentrations (C_i, µmol CO_2) were measured in salt-treated *Aster* (0%, 1.5%/257 mM, and 3%/513 mM NaCl) and *Sesuvium* (0%, 2.5%/428 mM, and 5%/856 mM NaCl) plants for up to 10 days. **a** and **c** Data collected from *Aster*. **b** and **d** Data collected from *Sesuvium*. The measurements were made always in the morning between 9-10 am three hours after switching on the additional light. The calculation of the assimilation rate is based on the difference of the gas concentration and the airflow rate (Sandra 2002). The measurements were made in the leaves from different plants for each of the NaCl concentrations.

The leaf CO_2 concentrations C_i (µmol CO_2) in both *Aster* and *Sesuvium* were reduced from about 350 at the beginning to 215 µmol at the end of the experiment in 3%/513 mM NaCl *Aster* or from 330 to only 50 µmol CO_2 in *Sesuvium* 5%/856 mM NaCl (Fig. 4c and d). Control plants in both species almost maintained their leaf CO_2 concentrations. In *Aster* 1.5%/257 mM NaCl watered plants were initially affected due to NaCl stress but later a stabilisation along with the control was observed. *Sesuvium* 2.5%/428 mM NaCl plants had a lower CO_2 concentration than the control, but the concentration did not change during the experiment.

The differences in transpiration rate (E) in *Aster* and *Sesuvium* between the control and NaCl treated plants started to emerge at day 3 of watering with NaCl (Fig. 5c and d). In *Aster* at day 10, the transpiration of 3%/513 mM NaCl watered plants was almost stopped having 1.29 mol H_2O m^{-2} s^{-1}whereas 1.5%/257 mM plants stabilized in the same range of the control at 5.08 mol H_2O m^{-2} s^{-1}. The transpiration rate in *Sesuvium* was half of the value of *Aster*. The transpiration rate remained almost stable in the control plants having normal or higher transpiration rate than NaCl treated plants. Though 2.5%/428 mM NaCl treated plants seemed to be developing towards stabilization after initial fluctuations having the value of 1.480 mol H_2O m^{-2} s^{-1}, but 5%/856 mM plants had almost stopped transpiration with a value of 0.05 mol H_2O m^{-2} s^{-1}. The water use efficiency (WUE) had been expressed as the result of CO_2-saturated photosynthetic assimilation rate (A) divided by the transpiration rate (E). In both *Aster* and *Sesuvium* a direct correlation between transpiration rate and water use efficiency was observed (Fig. 5e and d). Both plants had higher WUE in 3%/513 mM NaCl or 5%/856 mM NaCl watered plants, respectively, compared with their respective controls. Stomatal conductance (g_s) though initially heavily unsettled resembled during the experimental course the results of transpiration rate and WUE in both *Aster* and *Sesuvium* by showing closing of the stomata in plants treated with high NaCl compared to their controls (Fig. 5a and b).

3.2.4 Photosynthetic characteristics: chlorophyll fluorescence, chlorophyll, and carotenoids

In the *Aster* plants investigated the total chlorophyll contents calculated on the basis of fresh weight constantly increased during the experiment in an age and salt dependent manner. However, the leaves of *Aster* plants treated with NaCl contained always higher amounts of chlorophyll than untreated plants (Balasubramanian et al., in preparation). The chlorophyll a

to b ratios indicated a proportionally higher increase of chlorophyll b than chlorophyll a (Table 3).

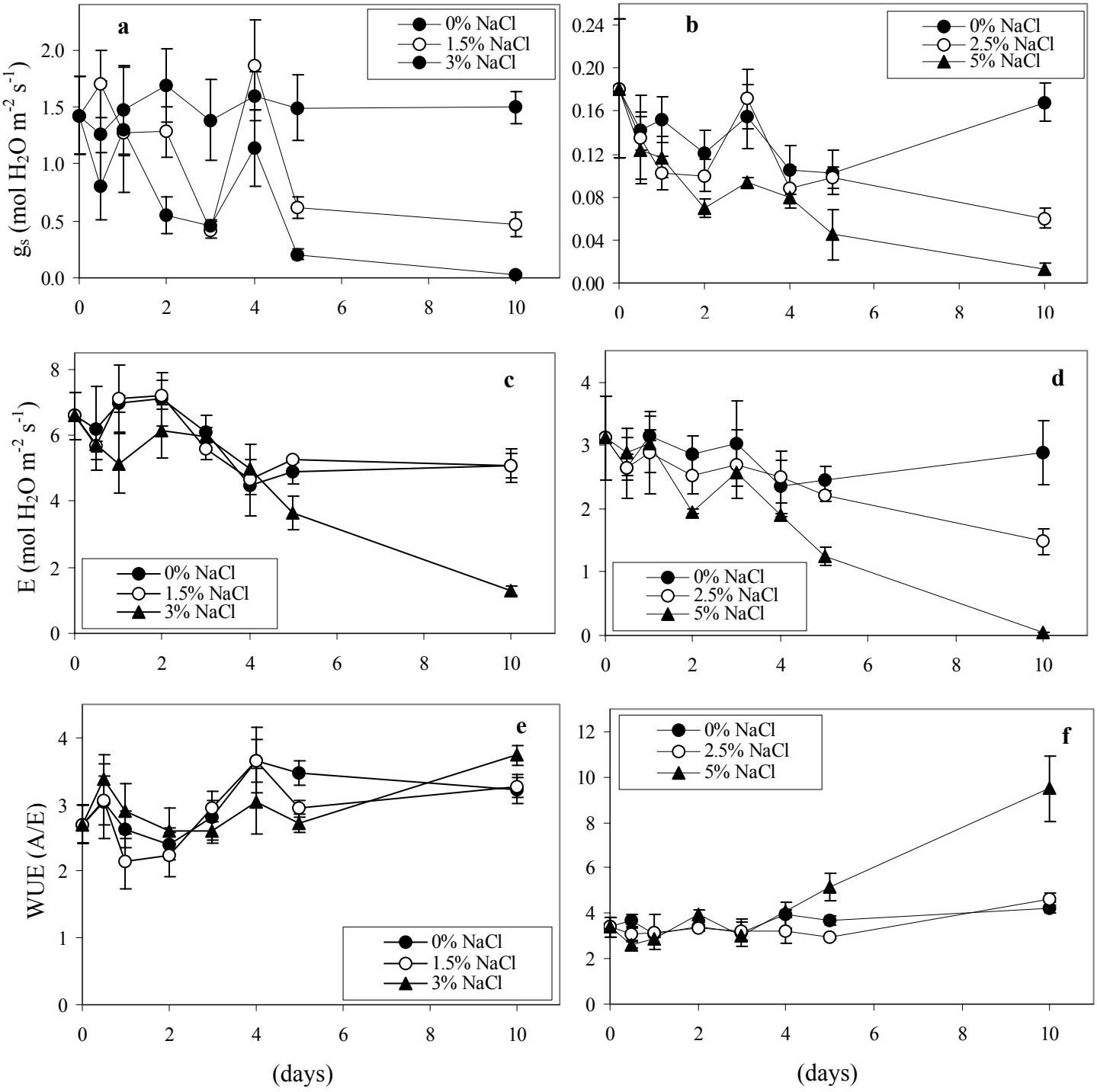


Fig. 5a-f Interrelated photosynthetic parameters of stomatal conductance (g_s, given in mol H_2O m^{-2} s^{-1}), transpiration rate (E, given in mol H_2O m^{-2} s^{-1}), and water use efficiency (WUE, given as CO_2 assimilation rate (A)/transpiration rate (E)). **a, c, and e** Data collected from *Aster*. **b, d, and f** Data collected from *Sesuvium*. Stomatal conductance (g_s) in mol H_2O m^{-2} s^{-1}, transpiration rate (E) in mol H_2O m^{-2} s^{-1} and water use efficiency (WUE) obtained from CO_2 assimilation rate (A) divided by the transpiration rate (E) in the leaves were measured using a LCi portable photosynthesis system in the greenhouse.

In *Sesuvium* the chlorophyll content clearly decreased on the third day after beginning of the salt treatment whereas the age or longer term salt treatment do not show strong effects on the chlorophyll content. Along with chlorophyll, carotenoid contents were also analysed because of its importance in the protection of PSII.

Table 3 Chlorophyll a/b and carotenoids content in *Aster* and *Sesuvium* watered with 0%, 1.5%/257 mM, and 3%/513 mM or 0%, 2.5%/428 mM, and 5%/856 mM NaCl, respectively, in the watering solution and harvested every second day starting one day after beginning of the treatment. FW, fresh weight.

	Chlorophyll a/b in *Aster*			Chlorophyll a/b in *Sesuvium*		
	NaCl			NaCl		
time	0%	1.5%	3%	0%	2.5%	5%
day 1	3.88	3.06	3.63	3.71	4.24	4.14
day 3	3.87	3.54	3.12	4.10	4.06	5.01
day 5	3.81	3.51	3.33	3.69	4.94	4.97
day 7	3.52	4.02	3.33	3.33	4.42	4.94
day 9	3.54	3.58	3.13	3.95	4.27	4.39
	Carotenoids in *Aster* ($\mu g\ g\ FW)^{-1}$			Carotenoids in *Sesuvium* ($\mu g\ g\ FW)^{-1}$		
	NaCl			NaCl		
time	0%	1.5%	3%	0%	2.5%	5%
day 1	156±17	234±2	204±14	122±13	145±8	198±13
day 3	139±4	217±17	220±9	119±3	147±12	126±9
day 5	191±26	268±12	227±4	159±8	154±7	190±12
day 7	189±9	268±9	320±7	151±4	187±10	139±3
day 9	229±21	273±13	284±9	153±11	144±10	184±4

In *Aster* a slight increase in carotenoids could be seen in the 3%/513 mM NaCl watered plants compared to the control, whereas no changes were observed in *Sesuvium* with and without NaCl in the watering medium (Table 3).

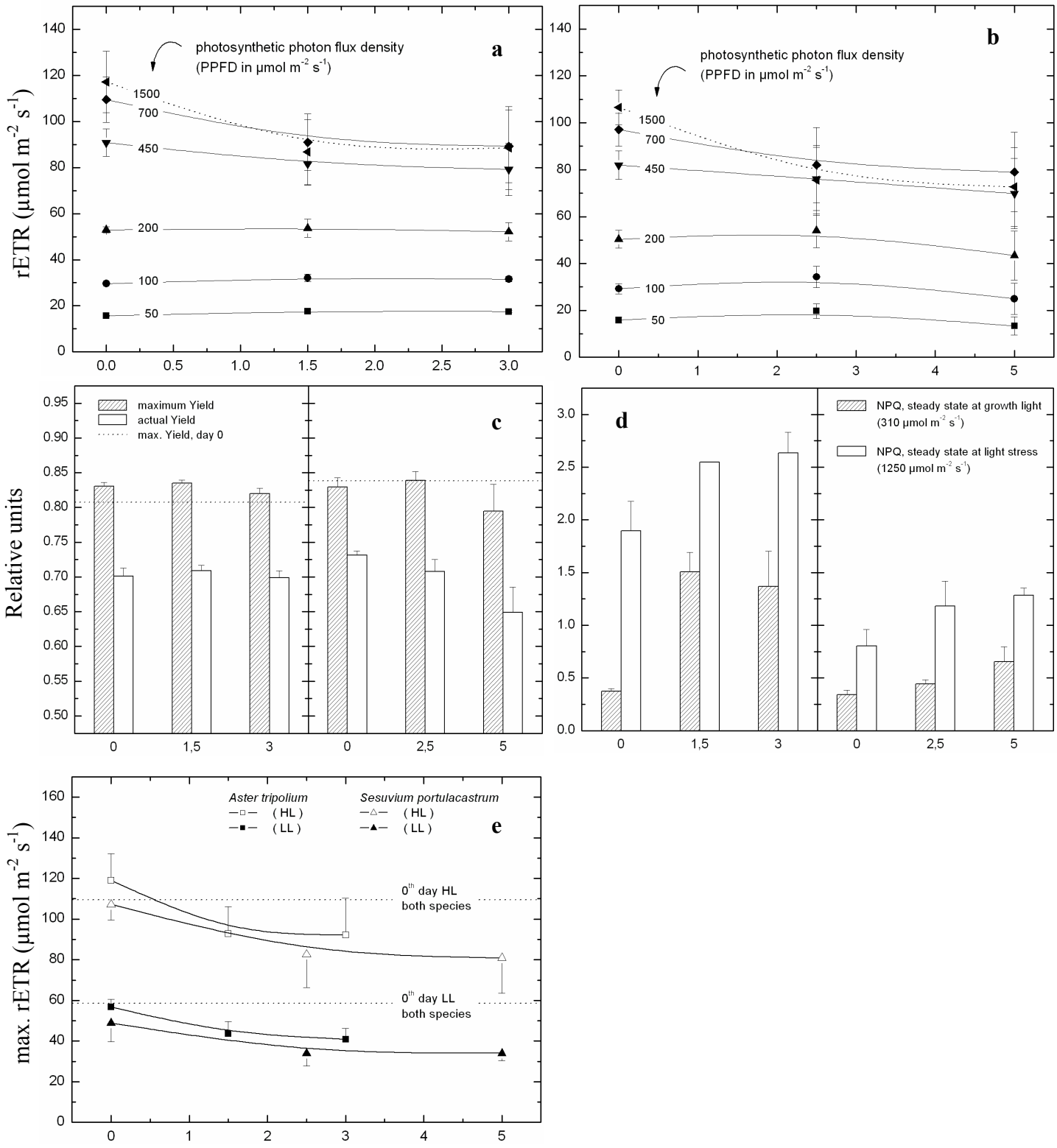


Fig. 6a-e Chlorophyll fluorescence measurements. All the measurements were made in *Aster* and *Sesuvium* treated with 0%, 1.5%/257 mM, and 3%/513 mM or 0%, 2.5%/428 mM, and 5%/856 mM NaCl, respectively, in the watering solution and measured at the start and end of the experiment lasting for 10 days in the climatic chamber having the conditions of 21±2°C, 60% humidity, and a 12 h light/12 h dark period. **a** Relative electron

transport (rETR) in *Aster* at different actinic light intensities. **b** Relative electron transport (rETR) in *Sesuvium* at different actinic light intensities. **c** Maximum yield and actual yield in *Aster* (left partition in the graph) and *Sesuvium* (right partition in the graph). **d** Non-photochemical quenching (NPQ) in *Aster* (left partition in the graph) and *Sesuvium* (right partition in the graph) at 310 and 1250 µmol m^{-2} s^{-1}. **e** Maximum relative electron transport rate (max. rETR) in *Aster* and *Sesuvium* at 50 and 310 µmol m^{-2} s^{-1}. Modulated Chl fluorescence measurements were made on attached leaves using a Mini-PAM portable fluorometer (Walz, Effeltrich, Germany) connected to a notebook computer with data acquisition software (WinControl 1.93). The experimental protocol of Demmig-Adams et al., (1996) was taken as the base model. Actinic light was provided by KL-1500 lamps (Schott, Stafford, UK). The irradiance on the leaf surface was adjusted to growth light of approximately 50, 310, or 1250 µmol m^{-2} s^{-1}, respectively, for low light, high light, or light stress. F_m was measured after 15-20 min of dark adaptation. Light intensity was measured by a microquantum-sensor which was measured against a calibrated external quantum sensor (LI-250, LI-1905A, LiCOR, Nebraska, USA) mounted in the leaf-clip holder (Model 2030-B, Walz, Germany). The photosynthetic yield i.e. the actual PSII efficiency (Φ_{PSII}) was calculated according to Genty et al., (1989). Non-photochemical quenching (NPQ) was calculated as $(F_m/F_m')-1$ according to Bilger and Björkman (1990). Relative electron transport rate (rETR) was calculated following the formula: $rETR = \Phi_{PSII} * PPFD * 0.5 * 0.84$, whereas PPFD refers to photosynthetic active radiation in µmol m^{-2} s^{-1}, 0.5 accounts for an equal distribution between the two photosystems and 0.84 was an average of absorbance of a leaf. Measured values (eg. max. rETR) were analysed and fitted using the model described by Eilers and Peeters (1988).

Additionally experiments were conducted to analyse the changes of the efficiency and capacity in the PSII photochemistry in *Aster* and *Sesuvium* grown in varying NaCl concentrations and light intensities. Results of the relative electron transport rate (rETR) showed no significant changes at varying actinic light intensities from 50 to 700 µmol m^{-2} s^{-1} in both *Aster* and *Sesuvium* when watered with NaCl in the growth medium whereas at 1,500 µmol m^{-2} s^{-1} a small decrease in rETR was observed (Fig. 6a and b). Almost similar results were echoed in the measurement of maximum yield and the actual yield in both plants at growth light of 310 µmol m^{-2} s^{-1} (Fig. 6c). In *Sesuvium* there seemed to be tendency of decrease in both actual and maximum yield at the highest NaCl concentration of 5%/856 mM. Figure 6d shows the changes in non-photochemical quenching (NPQ) in *Aster* and *Sesuvium* grown at 310 (high light) and 1,250 (moderate light stress) µmol m^{-2} s^{-1} actinic light. Two different results were derived from NPQ analysis. First, the results show that both plants had linearly increasing levels of NPQ with increasing NaCl concentrations. Second, the results show that *Sesuvium* had almost half the NPQ value compared to *Aster* but only under salt stress. As shown in Figure 6e, the maximum electron transport showed changes from day 0 to day 10, but there were no significant differences observed between both species, also when grown at low light or high light.

3.2.5 Determination of ATPase activities

In *Aster* and *Sesuvium* all the three major types of ATPases had been analysed namely P-, V-, and F-type H^+-ATPases.

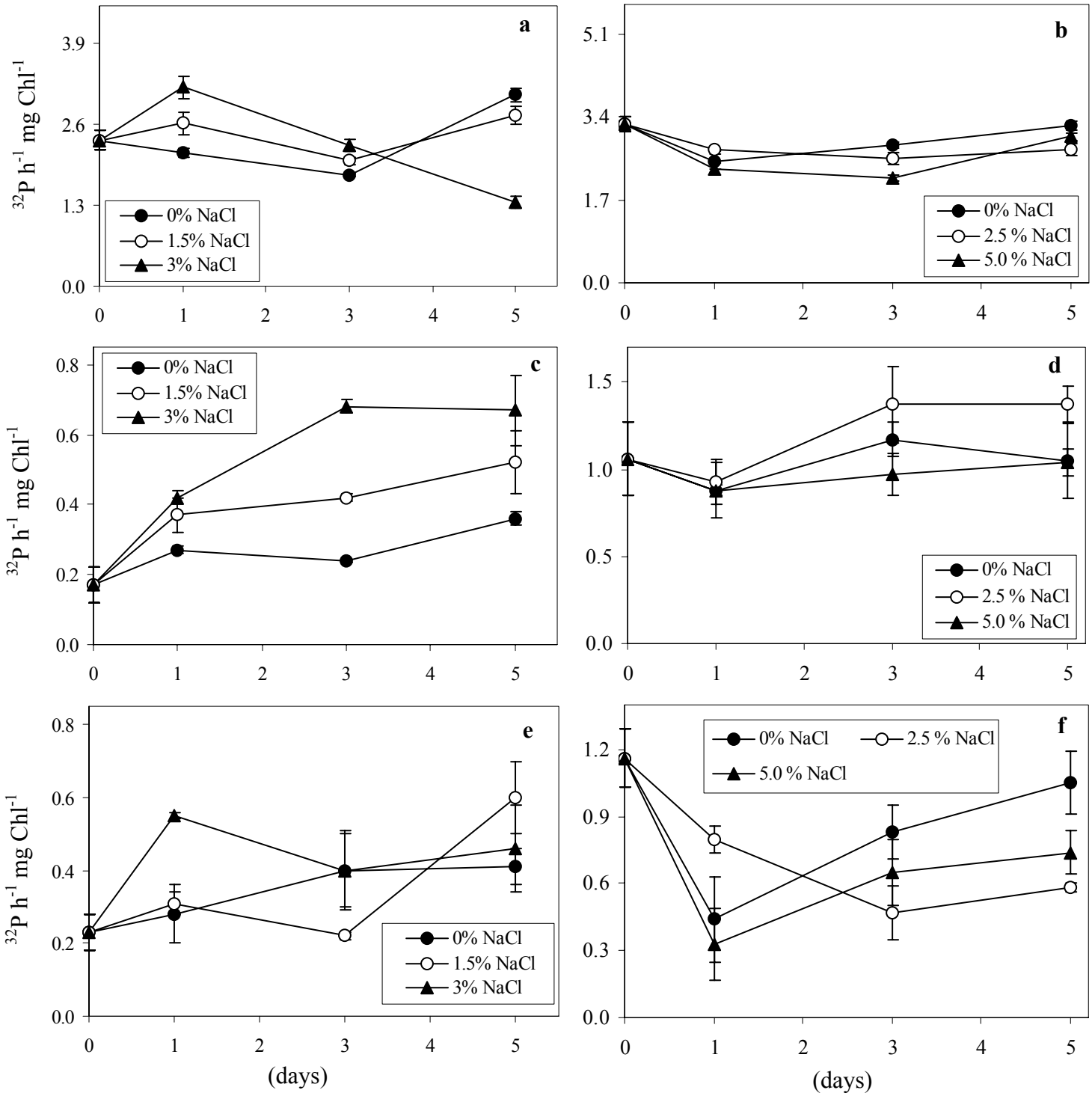


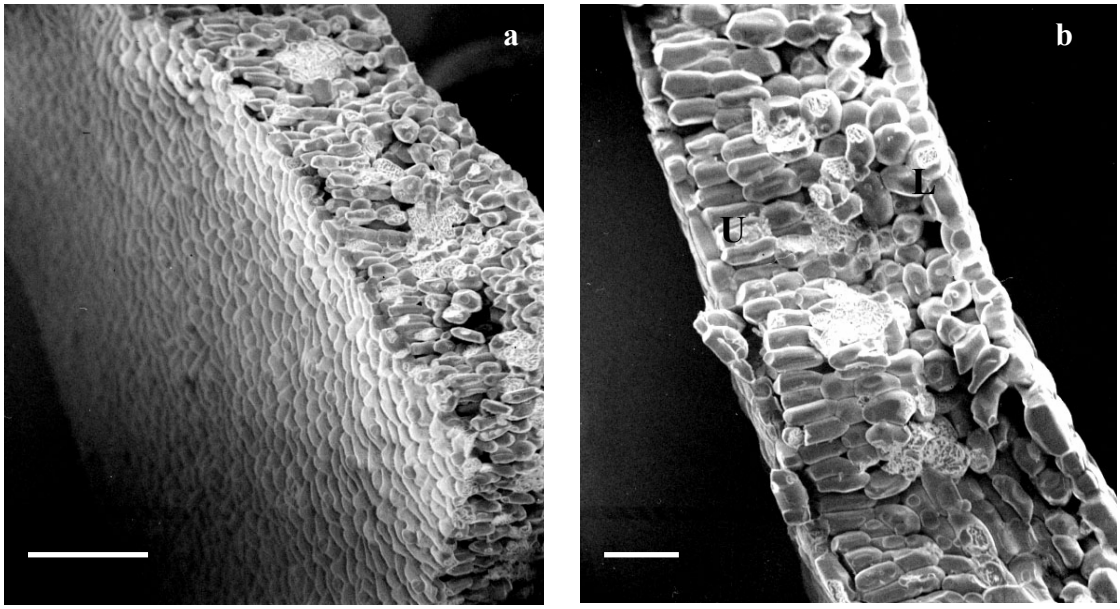
Fig. 7a-f Leaf ATPase activity in *Aster* and *Sesuvium* treated with 0%, 1.5%/257 mM, and 3%/513 mM or 0%, 2.5%/428 mM, and 5%/856 mM NaCl, respectively, in the watering solution and measured on day 0, 1, 3, and 5 with the experiment lasting for 10 days. **a** and **b** Plasma membrane-H^+-ATPase activity data measured from *Aster* and *Sesuvium*. **c** and **d** Chloroplast and mitochondrial F-H^+-ATPase activity data collected from *Aster* and

Sesuvium. **e** and **f** Vacuolar H^+-ATPase activity data collected from *Aster* and *Sesuvium*. The preparation of cell homogenates and the determination of ATPase activities were done according to Koyro et al. (1993) with some modifications. To differentiate between different ATPases following inhibitors were added to a final concentration of 1 mM V_2O_5 to inhibit P-type ATPases, 40 mM KNO_3 to inhibit V-type ATPases or 5 mM NaN_3 to inhibit F-type ATPases. The pH of the inhibitor solutions was adjusted to pH 7.4.

In *Aster* there was an initial increase in P-ATPase activity in 3%/513 mM NaCl watered plants on day 1 but then the activity decreased and never recovered, but in 1.5%/257 mM NaCl treated plants the activity remained almost unchanged after an initial NaCl effect (Fig. 7a). There were no significant differences between the control and NaCl treated *Sesuvium* plants in the P-ATPase activity (Fig. 7b). There were clear and strong differences between the control and NaCl treated *Aster* concerning F-ATPase activity localized in chloroplasts and mitochondria (Fig. 7c). The 3%/513 mM NaCl plants showed a three times and the 1.5%/257 mM NaCl treated plants a two fold higher activity compared to the control. In *Sesuvium* there are no significant changes in the F-ATPase activity between the control and NaCl treated plants (Fig. 7d). In both *Aster* and *Sesuvium* no significant increase or decrease in V-ATPase activities based in vacuoles could be observed (Fig. 7e and f).

3.2.6 Investigations in the leaf of *Aster* using SEM

Organelle investigations were carried out to know if there exist any special storage compartment in *Aster* other than the vacuoles in the cell. In our investigations no special structures could be traced in *Aster*. The representative figures of SEM are shown in Figure 8a to d. Due to technical difficulties *Sesuvium* leaves could not be analysed.



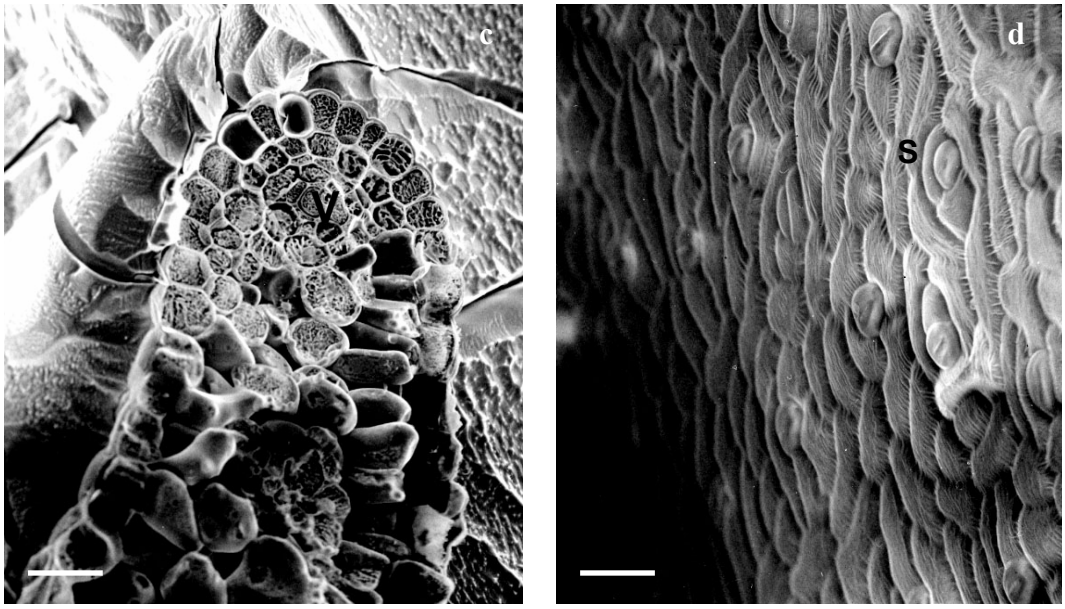


Fig. 8 Secondary electron image of a frozen, hydrated, and transversely fractured surface of a *Aster* leaf. **a** Leaf surface after the etching process. **b** transverse section showing upper (U) and lower epidermis (L), respectively. **c** Vascular bundle sheath marked as V. **d** Stomatal structures marked as S. The plants were grown in the nutrient solution having 3% NaCl concentration. The images were taken on day 5 of the experiment. For the images, freshly harvested leaf segments were mounted on specimen holders and quench frozen with nitrogen slush. The frozen leaf slices taken from the third node were fractured transversely, transferred, and investigated on the cold stage of an ETEC-Autoscan scanning electron microscope. Accelerating voltage was 10 kV and the beam current was 150 µA (Werner and Stelzer, 1990). Bars represent 100 µm for illustration a and 10 µm for illustrations b, c, and d. A representative picture is shown selected from several series made.

3.3 DISCUSSION

3.3.1 NaCl effects on the growth of *Aster* and *Sesuvium*

The direct effects of NaCl of reduced growth could be phenotypically seen in both plants *Aster* and *Sesuvium*. It has been reported before that when NaCl was less than 150 mM *Aster* cells could not sense it as salt stress or other stress adaptation mechanisms were used (Uno et al. 1998). *Aster* and *Sesuvium* both were having decreased fresh weight compared to the control plants in the soil culture (Fig. 1) as well as in hydroponic culture (data not shown). It has been reported that *Sesuvium* is a strict halophyte, growth stimulated by NaCl of 100-200 mM NaCl in the watering medium but were experimented up to 1000 mM NaCl (Mssedi et al. 2000).

3.3.2 Composition of ions during salt stress in *Aster* and *Sesuvium*

In our experiments *Aster* plants accumulated high levels of inorganic ions even at lower salinities in agreement with results obtained earlier (Shennan et al., 1987b). Na^+ ions accumulated with increasing NaCl concentrations whereas K^+ uptake showed no difference between salt treated plants and the control. Consequently the Na^+/K^+ ratio increased with increasing NaCl concentrations comparable to results from Ueda et al. (2003). The results of Na^+, K^+, and Cl^- analysis with SEM demonstrate an accumulation of ions in the mesophyll vacuoles of *Aster* already during short-term salt stress. In *Sesuvium* the Na^+ content in the leaves was very high compared to *Aster*. Here also Na^+ accumulation increased with increasing NaCl concentrations but K^+ accumulation remained at constant level in all treatments compared to the control plants. The Na^+/K^+ ratio resembled the values in *Aster* whereas the intake of Na^+ into *Sesuvium* is two fold higher than into *Aster*. Looking at the ion accumulation data, one can presume *Aster* as an Na^+ excluder and *Sesuvium* as a Na^+ includer, at least in the short-term. Interestingly, even the control plants watered with tap water accumulated some NaCl to maintain normal growth. When compared to the ion concentrations in the growth substrate determined by inductively coupled plasma analysis, the results reflected the Na^+ concentrations in the leaves of *Aster* and *Sesuvium* (data not shown). The reported accumulation of ions in Aster and Sesuvium leaves resembling a true halophyte was in good agreement with the experiments using halophytic species *Suaeda salsa* (Kefu et al., 2003) and *Artimisia anethifolia* (Lu et al., 2003).

Another indication of ion uptake by *Aster* and *Sesuvium* has been reflected through osmotic adjustment. Due to increasing amount of Na^+ in line with increasing NaCl concentrations of the watering medium, the osmolarity level also increased in direct correlation in both *Aster* and *Sesuvium*. This result agrees with the argument of Ueda et al. (2003) that *Aster* has mechanisms to accumulate Na^+ and Cl^- in vacuoles and osmolytes in cytosol to maintain the osmotic potential in the cell. However, it is not clear which osmolytes or compatible solutes, amino acids or sugars contribute to the increase in osmolarity Currently, we are following several possibilities to analyse the putative counter ions to balance the Cl^- amounts, presumably organic acids.

3.3.3 Water relations during salt stress in *Aster* and *Sesuvium*

Shennan et al. (1987a) reported that *Aster* did not show increased succulence at high salinity, leaf fresh weight to dry weight ratio declined, whilst leaf fresh weight per unit area remained

constant. Those observations were in good agreement with the degree of succulence, which remained almost unchanged between control and NaCl treated *Aster*. The relative water content (RWC) of *Aster* had no significant changes due to NaCl treatment. Ueda et al. (2003) pointed out that *Aster* did not suffer from tissue dehydration in highly salinized environments and , however, the plant did not reveal drought tolerance. *Sesuvium* also had the same effect as *Aster* in RWC. An interesting aspect might be the marginal decrease in the degree of succulence in *Sesuvium* 5%/856 mM NaCl watered plants compared to their control. Succulent halophytes has been normally hypothesized to have its succulence increased due to the high uptake of water to maintain their osmotic potential risen due to the uptake of ions from the external medium. Inspite of decrease in the fresh weight, the RWC remained almost constant at 90% (data not shown) and the water supply to the shoot seems to flow uninterrupted as reported also for *Spartina townsendii* (Koyro, 2002).

3.3.4 Effects of NaCl on chlorophyll and carotenoid contents in *Aster* and *Sesuvium*

There was a strong increase in total chlorophyll content in *Aster* whereas no significant changes could be observed in *Sesuvium* (Balasubramanian et al., in preparation). The chlorophyll a to b ratio decreased slightly with increasing NaCl concentrations in *Aster*, whereas in *Sesuvium* the ratio slightly increased. One could speculate that the antennae pigments need to be more effective to deliver sufficient energy to deal with the energy consuming adaptations to salt stress. *Aster* shows high resistance to salt stress by increasing chlorophyll contents to have higher light harvesting capacity and hence higher photosynthesis, whereas in *Sesuvium* no significant changes in chlorophyll making it not susceptible to salt. There were no significant changes in the total carotenoid contents neither in *Aster* nor *Sesuvium*. With no change in carotenoids it could be assumed that there is no need for photoprotection with significant increase of total chlorophyll in *Aster*. This view has been expressed also by Fedina et al. (2003). However, the analysis of the composition of the carotenoid pool would be helpful for further conclusions. Obviously chlorophyll biosynthesis and stability of the two plant species have been adapted in different ways to high salinity. The result of no significant changes in the carotenoid contents has been expressed by Lu et al. (2003), but they have also reported an increase in the carotenoid contents in the cleary day compared to cloudy day. This implies that individual composition of xanthophylls xycle pigments needs to be analysed to know whether the increase in carotenoids is due to light stress or salt stress.

3.3.5 Chlorophyll fluorescence during salt stress

The simulated relative electron transport rates (rETR) based on different measured actinic light intensities were analysed at varying NaCl concentrations in *Aster* and *Sesuvium* to understand the photosynthetic capacity and the effects on PSII photochemistry. There were no significant differences between both halophytes *Aster* and *Sesuvium* after 10 days of experimental period under varying growth light from 50 to 1,500 µmol m^{-2} s^{-1}. At the same period of time and in the same plants the maximum electron transport rate (max. rETR) revealed no significant changes with respect to the NaCl concentration and the actinic light combination of 50 (low light) and 310 (high light) µmol m^{-2} s^{-1}. More interesting are the results concerning the yields and the non-photochemical quenching (NPQ). The maximum yield in both *Aster* and *Sesuvium* had the value of around 0.8 relative units, which is regarded as base value in higher plants as an indicator of well functioning photosynthesic processes (Björkman and Demmig, 1987). The actual yield seemed to be affected slightly in both *Aster* and *Sesuvium* at highest NaCl concentrations of 3%/513 mM and 5%/856 mM, respectively. The most exciting part of the chlorophyll fluorescence investigation up to now here is the outcome of the determination of NPQ. Results of the NPQ values in *Sesuvium* were almost half the NPQ values determined in *Aster* at the normal growth light (high light) (Fig. 6d). The second interesting outcome is the increase in NPQ with increasing NaCl concentrations grown under actinic light of 310 µmol m^{-2} s^{-1} whereas under light stress conditions (1,250 µmol m^{-2} s^{-1}) the NPQ values were almost two fold higher in both plants. Since PSII is believed to play a key role in the response of photosynthesis to environmental perturbations (Baker, 1991), the effects of high salinity on PSII had been investigated extensively. Although much effort was invested to elucidate the causes of the decreased photosynthetic capacity, the underlying mechanism is still unclear. One probable thought put forward by Osmond (1981) was, that stomatal closure at peak irradiance deprives leaves of CO_2 The shortage of CO_2 might lead to the development of reactive oxygen species by the photosynthetic apparatus when the photochemical energy cannot be dissipated in an orderly manner. The chlorophyll fluorescence results of *Aster* and *Sesuvium* reaffirms with our pigmental variations that both plants respond with different strategies to high salt. The analysis of xanthophylls cycle compounds would give us a picture which could solve the NPQ outcome. At this point, thinking of energy linking process, it should be noted that F-ATPase is increasing with increasing NaCl in *Aster* but not in *Sesuvium*. Therefore it can be proposed that ATP consumption in *Aster* is higher compared to *Sesuvium*. The protein complexes could also be

more properly protected in *Sesuvium* by higher osmolyte production as evident from osmolarity values given in this investigation.

3.3.6 Leaf CO_2 assimilation rate and intercellular concentration affected during NaCl stress

Salinity strongly influenced the photosynthetic assimilation rate (A) and other gas-exchange parameters in both *Aster* and *Sesuvium*. Increasing NaCl leads to a similar decrease of the net photosynthesis in both species. The decrease of A is accompanied by a decrease in C_i. *Aster* is obviously a C_3 plant because even at high salinity at any time C_i is above 200 µmol CO_2 so that RubisCO can still work. When analysed using immunoblotting the RubisCO steady-state levels did not show any significant changes (data not shown). *Sesuvium* seemed to switch to C_4 or CAM metabolism. During the time course of the experiment the C_i was lowered down to 50 µmol CO_2 and therefore phosphoenolpyruvate carboxylase (PEPC) needs to be the first CO_2 fixating enzyme. Analysis of organic acids and enzymes involved in the CAM shift is in progress.

3.3.7 Stomatal conductance, transpiration rate, and water use efficiency (WUE)

In both *Aster* and *Sesuvium,* decreased stomatal conductance diminished transpiration rates, and thus WUE improved. An explanation could be derived for these results from the argument of Everard et al. (1994) in which the authors proposed reduced transpiration rates and decreased stomatal conductance will tend to reduce the rate of salt loading into the leaves and would increase leaf longevity by maintaining salts at subtoxic levels longer than would occur if transpiration rates were not diminished. At higher salinities in both *Aster* and *Sesuvium*, stomatal conductance was severely reduced along with impairment of carboxylation capacity. Probably both stomatal and non-stomatal limitation contribute to this. It can be explained that stomatal factors apparently effectively limited *A* at intermediate salinities, and non-stomatal limitations prevailed under more severe salinity stress (Everard et al., 1994).

3.3.8 Effect of NaCl on the P-, F- and V- H^+-ATPase activities

Plasma membrane (PM), vacuolar, and mithochondrial/chloroplast H^+-ATPases activities in *Aster* and *Sesuvium* were affected in response to NaCl in the growth medium. A transient increase for PM H^+-ATPase activity with a maximum after 1 day watering with 3% NaCl in

the case of *Aster* was observed followed by a clear decrease up to day 5. The changes in activity are accompanied by changes in the PM H^+-ATPase protein levels when analysed with immunoblotting (data not shown). For the *Sesuvium* PM H^+-ATPase activity no significant changes could be noticed between control and plants with NaCl in the growth medium. One could speculate that both plants have an important strategy in their roots when confronting with NaCl and make sure fewer ions reaches the leaves.

The *in vitro* import of cytoplasmically synthesized proteins into chloroplasts has been demonstrated and it requires F-ATPase (Pain and Blobel, 1987). In *Aster* the F-ATPase activity was clearly stimulated by the NaCl in the growth medium. NaCl treated plants always had higher activity than the control plants, whereas in *Sesuvium* no significant changes could be observed. Kanazawa and Kramer (2002) proposed that F-ATPase assumes an important role in regulating adjustments of the photosynthetic system to environmental conditions by varying the relationship of photosynthetic electron transport to NPQ (Non-photochemical quenching). Another specific factor was a suggestion that sulfoquinovosyldiacylglycerol, plant sulfolipid localized in the thylakoids might play a role in stabilizing the protein complexes in chloroplast membranes such as PSII (Minoda et al., 2003) and CF_0-CF_1 ATPase molecules (Pick et al., 1985).

The tonoplast localized V-ATPase did not show any significant changes due to NaCl stress in both plants. The active transport process is important for the compartmentation of metabolically toxic Na^+ ions inside the vacuole. The main strategy for the salt marsh plant *Suaeda salsa* L. was reported to be the up-regulation of V-type ATPase activity in tonoplast vesicles but by an increase in V-type ATPase protein amount (Wang et al., 2001). Another adaptation to salt might be an increase in the volume of the vacuole due to accumulation of ions and osmolytes (Mimura et al., 2003). The accumulation of Na^+ and Cl^- without the highly active V-ATPase in *Aster* and *Sesuvium* is hard to explain and remains an open question. In preliminary microarray results using the heterologous system of *Arabidopsis* EST clones and *Aster* probes the expression of a homologue to the V-type H^+-ATPase from *Arabidopsis* was highly induced at high salt (data not shown). *Sesuvium* essentially needs NaCl for an optimal growth. It seems as if NaCl is used for several factors, such as increasing the osmotic potential. It might give an impression as if a part of the energy is saved because of the use of NaCl for osmotic factors. However, *Sesuvium* is a very slow reacting plant as it could be seen even with the growth and morphological changes due to salt stress with the changes probably take place in a long term. The active transport process is important for the compartmentation of metabolically toxic Na^+ ions inside the vacuole.

3.3.9 Strucural variations in *Aster* leaves due to salt stress

The exploration for specially developed or formed storage organs for ions in *Aster* was also investigated using scanning electron microscope (SEM). Although some species have salt glands or can develop succulence to increase their vacuolar capacity for salt accumulation, most halophytes do not have any obvious morphological adaptation (Very et al., 1998). Initial search revealed no peculiar formations when analysed using cryo-preserved leaf samples. Though a more careful approach using transmission electron microscopy (TEM) for analysis in the cell organelles (cell wall and chloroplast) is underway before making any final conclusions about the ultrastructural role in compartmentation of ions. Increasing the vacuolar volume could potentially protect the cytoplasm by decreasing the cytoplasmic volume during the initial phases of salt stress (Mimura et al., 2003). Together with accumulation of ions in the cells without prolific activities of both PM and vacuolar H^+-ATPases and the above said vacuolar volume increase motivated us to search for if any peculiar structures for the ion accumulation in *Aster* cells. At this point due to technical difficulties in the sample preparation it was not possible to reproduce the initial results (data not shown). Unfortunately, the analysis did not reveal any novel morphological structure for the storage of ions. One could think about ion accumulating cell walls which prevent toxic concentrations within the cells. In summary it can be concluded ion that both *Aster* and *Sesuvium* employ different strategies during short-term adaptation to high salt not only because the latter being a CAM and the former a C_3 plant. Other factors namely different way of ions accumulation and their concentration leveis, different protection levels for the photosystem and reaction centres, varied ATPase activities and still unknown factors out of this paper's scope.

4 Sulfolipids in halophytes and glycophytes during salt stress

4.1 INTRODUCTION

Recently, the whole biosynthetic pathway of SQDG was elucidated in higher plants: The SQDG biosynthesis takes place in plastids and is catalysed by two enzymes called sulfoquinovosyltransferase 1 (SQD1) and sulfoquinovosyltransferase 2 (SQD2). SQD1 catalyses the incorporation of SO^{-}_{3} into UDP-Glucose forming the head group UDP-sulfoquinovose. SQD2 catalyses the second and final reaction of SQDG formation by addition of diacylglycerol (DAG) to the head group (Yu et al., 2002).

It was shown previously that environmental and nutritional factors play a role in the synthesis of SQDG, like water deficit (Muller and Santarius, 1978), temperature (Quartacci et al., 1995; Sato et al., 2003), sulfate deprivation (DeKok et al., 1997), and phosphate starvation (Essigmann et al., 1998; Yu et al., 2002). There are several reports describing the effect of salinity on the SQDG contents in glycophytes (Kuiper et al., 1974; Pearcy, 1978; Stuiver et al., 1981; Kettunen et al., 1996). In a salt tolerant genotype of *Beta vulgaris* L. the SQDG content increased during NaCl treatment (Stuiver et al., 1981). Thus formation of SQDG may be one important aspect of strategies involved in salt tolerance.

It was suggested that SQDG might play a role in stabilizing the protein complexes in chloroplast membranes such as photosystem II (PSII) (Minoda et al., 2003) and CF_0-CF_1 ATPase molecules (Pick et al., 1985). Supporting evidence was given by the fact that ATPase activity was inhibited when SQDG tightly bound to the CF_0-CF_1 complex of chloroplast ATPase was removed (Barber and Gounaris, 1986). In a recent report it was shown that a complex between NADPH:protochlorophyllide oxidoreductase, which catalyses the strictly light-dependent step of chlorophyll biosynthesis in angiosperms, and its substrates protochlorophyllide a and b could be only reconstituted in the presence of SQDG (Reinbothe et al., 2003).

The sea aster *Aster tripolium* L., Asteraceae, grows in diverse environments especially in the salt marshes along the coastlines in temperate regions but also in other inland places with saline soils (Wagenvoort et al., 1989). *Sesuvium portulacastrum* L. belongs to the Aizoaceae family and is located naturally in the tropical and subtropical coastal and brackish areas around the world. In addition *Thellungiella halophila* Mey., Brassicaceae, a halophyte growing around the east coast of China, and the glycophyte *Arabidopsis thaliana* Heynh. (ecotype Wassilewska), Brassicaseae, were chosen for SQDG analysis.

The experimental plants were watered with different NaCl concentrations and subsequently the SQDG contents were determined. For the quantification of SQDG in a sensitive and reproducible way several methods according to different authors were tested (Murakami-Murofushi et al., 1985; Benning and Somerville, 1992; Archer et al., 1997) and finally a combination of all three methods was applied. The results of the SQDG quantification of SQDG in *Aster*, *Sesuvium, Thellungiella*, *and Arabidopsis* grown under varying NaCl concentrations are shown. In addition chlorophyll a and b contents were analysed during the salt treatment experiments to know if there exist any correlation with SQDG contents. During watering with different NaCl containing solutions SQDG contents were always increased in *Aster*, *Sesuvium,* and slightly in *Thellungiella* but not in *Arabidopsis*. Because the SQDG contents increased only in halophytes, we got interested to know their SQDG fatty acid profiles and degree of unsaturation in *Aster* and *Sesuvium* to know the possible contribution of unsaturated fatty acids in SQDG towards salt tolerance in these halophytes. Fatty acids unsaturation profiles and identification of a rare molecular species of SQDG by LC-MS in *Aster* and *Sesuvium* are described.

4.2 RESULTS AND DISCUSSION

4.2.1 Effect of salt treatment on the phenotype of experimental plants

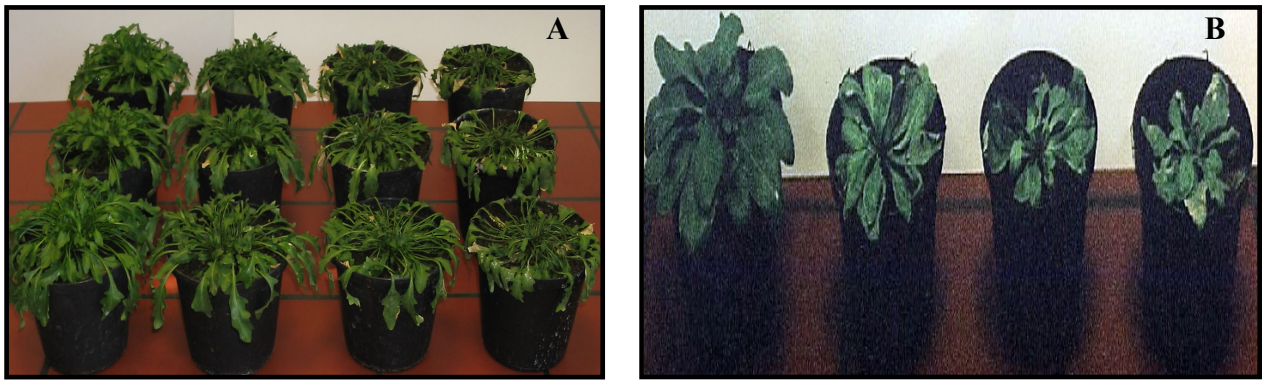


Fig. 1. Effect of NaCl on the growth of *Thellungiella halophila*, and *Arabidopsis thaliana* plants cultivated with different salt concentrations. (A) *Thellungiella* (from the left to the right, 0 mM, 100 mM, 300 mM, and 500 mM NaCl), (B) *Arabidopsis* (from the left to the right, 0 mM, 50 mM, 75 mM, and 100 mM NaCl) in the watering solution for up to 10 days. Conditions in the greenhouse were a 16 h light/8 h dark rhythm at temperatures of 23°C/21°C with humidity around 55%. When necessary, additional light was switched on for 16 h per day to obtain a constant quantum fluence rate of 300 µmol $m^{-2}s^{-1}$. The plants showed decreased growth rates with increasing salt concentrations.

According to published results *Aster* plants can grow at salt concentrations up to 500 mM (Kerstiens et al., 2002), *Sesuvium* up to 1000 mM (Mssedi et al., 2000), *Thellungiella* up to 500 mM (Bressan et al., 2001), and *Arabidopsis* up to 200 mM (Shi et al., 2003). For the experiments done here the amounts of NaCl added to the watering solutions were determined in pre-tests to obtain similar effects for each plant species with respect to growth retardation and reduction of fresh weight increase (data not shown). All experimental plants showed reduced growth after 10 days of salt treatment with the NaCl concentrations indicated (Fig. 1A,B). *Aster* is able to grow on NaCl levels between 0 and 3% (513 mM), close to seawater salinity of 3.5% (refer to chapter 3). The succulent plant *Sesuvium* is more tolerant to NaCl and can grow up to 5% (856 mM) NaCl concentration with a comparable reduction in growth (refer to chapter 3). In *Thellungiella* the effect of salt stress was seen evidently in the plants watered with 500 mM NaCl (Fig. 1A) whereas in the closely related *Arabidopsis* (ecotype Wassilewska) growth was severely affected already below 100 mM NaCl (Fig. 1B). Both species in the Brassicaceae family showed necrotic cell death at the tips and margins of the older leaves. Except the succulent plant *Sesuvium* all experimental plants developed wilting symptoms during the experiment.

Table 1 Chlorophyll a and b contents in *Aster tripolium* watered with 0%, 1.5%/257 mM, and 3%/513 mM, *Sesuvium portulacastrum* watered with 0%, 2.5%/428 mM, and 5%/856 mM NaCl, *Thellungiella halophila* watered with 0 mM, 100 mM, 300 mM and 500 mM, and *Arabidopsis thaliana* watered or 0 mM, 50 mM, 75 mM, and 100 mM NaCl, and harvested every second day starting one day after beginning of the treatment.

NaCl conc.	Chlorophyll a and b in *Aster tripolium* (µg g FW)$^{-1}$				
	day 1	day 3	day 5	day 7	day 9
0%	734±82	677±28	955±87	942±132	1261±140
1.5%	1074±47	1174±44	1409±58	1297±83	1526±36
3%	1016±14	1196±36	1224±36	1725±40	1615±16
NaCl conc.	Chlorophyll a and b in *Sesuvium portulcastrum* (µg g FW)$^{-1}$				
	day 1	day 3	day 5	day 7	day 9
0%	481±31	445±62	525±12	687±15	635±69
2.5%	546±17	540±43	587±46	771±84	695±13
5%	807±49	464±18	784±35	778±22	727±22
NaCl conc.	Chlorophyll a and b in *Thellungiella halophila* (µg g FW)$^{-1}$				
	day 1	day 3	day 5	day 7	day 9
0 mM	1725±33	1747±116	1550±321	1594±193	1752±236
100 mM	2004±31	1849±82	2101±119	1928±58	2371±178
300 mM	1687±91	1806±272	1634±168	1817±242	1852±268
500 mM	1866±96	2146±105	1901±32	2142±122	2248±96
NaCl conc.	Chlorophyll a and b in *Arabidopsis thaliana* (µg g FW)$^{-1}$				
	day 1	day 3	day 5	day 7	day 9
0 mM	1352±89	1479±92	1400±66	1400±153	1505±201
50 mM	1388±201	1520±72	1535±53	1527±143	1681±52
75 mM	1466±49	1529±188	1535±179	1482±101	1508±136
100 mM	1486±67	1516±113	1670±28	1702±92	1597±132

In all *Aster* plants investigated the total chlorophyll contents calculated on the basis of fresh weight constantly increased during the experiment in an age and salt dependent manner (Table 1). However, the leaves of *Aster* plants treated with NaCl contained always higher amounts of chlorophyll than untreated plants. The chlorophyll a to b ratios indicated a proportionally higher increase of chlorophyll b than chlorophyll a (data not shown). One could speculate that the antennae pigments need to be more effective to deliver sufficient energy to deal with the energy consuming adaptations to salt stress. In *Sesuvium* the chlorophyll content was clearly decreased at the third day after beginning of the salt treatment whereas the age or longer term salt treatment do not show strong effects on the chlorophyll content. Therefore a short term effect due to the salt treatment could be observed. Neither in *Thellungiella* nor in *Arabidopsis* any significant changes in the chlorophyll contents could be detected.

4.2.2 Thin layer chromatography (TLC) of plant extracts

The combination of three TLC methods resulted in a complete and reproducible extraction (Benning and Somerville, 1992; Archer et al., 1997), a good separation (Murakami-Murofushi et al., 1985) and detection (Benning and Somerville, 1992) of SQDG from *Aster* leaves as shown in Fig. 2. SQDG can be identified by the parallel row having known amounts of a commercially available SQDG standard. The identity of plant SQDG has been proven mass spectrometry (MS) as will be described later. SQDG is well separated from monogalactosyldiacylglycerol (Fig. 2, line at the top) and phosphatidylethanolamine (Fig. 2, line at the bottom).

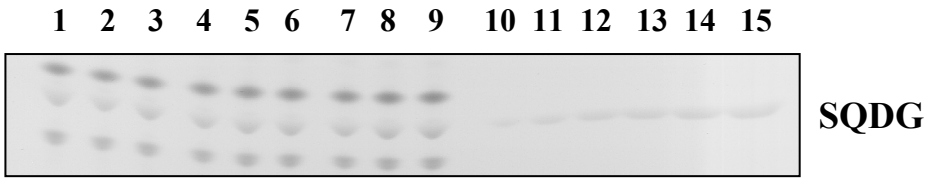


Fig. 2. Thin layer chromatography (TLC) of SQDG extracted from *Aster tripolium*. SQDG was extracted from 100 mg leaf material ground in liquid nitrogen with 300 µl of methanol:chloroform:water (65:25:4, v/v). After centrifugation the lower green phase was used for loading (10 µl/spot) on TLC plates. The lanes 1-9 represent *Aster* watered with different NaCl solutions for 1, 4, and 10 days: 1, day 1 0%; 2, day 1 1.5%; 3, day 1 3%; 4, day 4 0%; 5, day 4 1.5%; 6, day 4 3%; 7, day 10 0%; 8, day 10 1.5%; 9, day 10 3%. For identification and quantification a commercially available SQDG standard was loaded in lanes 10-15 (0.50, 1.0, 1.50, 2.0, 2.50, and 3.0 µg). The TLC plates were developed in chloroform:methanol:0.02% $CaCl_2$ in the ratio 60:40:9 (v/v) for 75 min (Murakami-Murofushi et al., 1985). The plates were exposed using sublimated iodine and the labelled spots were analysed through TINA 2.0 software package.

In comparison to the other methods tested the sensitivity of the analytical process could be improved because the extraction gave reproducible SQDG yields, the recovery rate was high and in a close range (85-87%), single individual SQDG spots could be separated with reproducible R_f-values of 0.73 by the chromatographic procedure, and the visualization with iodine gave lower background interference in comparison to $CuSO_4$ staining (Archer et al., 1997) which rose the reliability of density scanning and quantification using computer aided software. The SQDG quantification by TLC is still called a semi-quantitative method because the determination of spot size and intensity on thin layer plates is not as reliable as quantification of peaks produced by high pressure liquid chromatography (HPLC) or gas chromatography (GC) methods. Unfortunately, the development of a reliable HPLC method using small amount of leaf material was not successful so far using the published method of Norman et al. (1996) (data not shown).

4.2.3 Effect of NaCl on the sulfoquinovosyldiacylglycerol (SQDG) contents

The SQDG contents increased in the halophytes *Aster* and *Sesuvium* in comparison to the control plants watered without NaCl whereas in *Thellungiella* the SQDG contents did not change very much during the salt treatment (Fig. 3A-C). In *Sesuvium* the effect of NaCl treatment was more pronounced after a longer period of high salt treatment. The SQDG amounts after a 10-day-treatment with 5% NaCl in the watering solution was about twice as high as in the untreated control. In *Aster* the differences in SQDG contents between the control, and 1.5 and 3% NaCl in the watering solution were already substantiated after one day and did not change during the duration of the experiment. In the glycophyte *Arabidopsis* any significant differences could be observed between controls and salt-treated plants during the 10-day-exposure. The amount of SQDG in the plants investigated ranges from 140-580 µg per gram fresh weight. Though the amount of SQDG increased in *Aster* and *Sesuvium* leaves during salt treatment, the overall range of SQDG content is the same in glycophytes and halophytes. The increase in SQDG in *Aster* treated with high salt correlated positively with the increase in chlorophyll contents. Because salt ions are taken up by the roots one could speculate about the abundance and function of SQDG in root plastids during high salinity in halophytes. Therefore the roots of the same *Aster* and *Sesuvium* plants used for the SQDG analysis in leaves were collected and analysed. The amount of SQDG in roots ranges from 10-50 µg per gram fresh weight which is about 10% from the amounts determined in leaves.

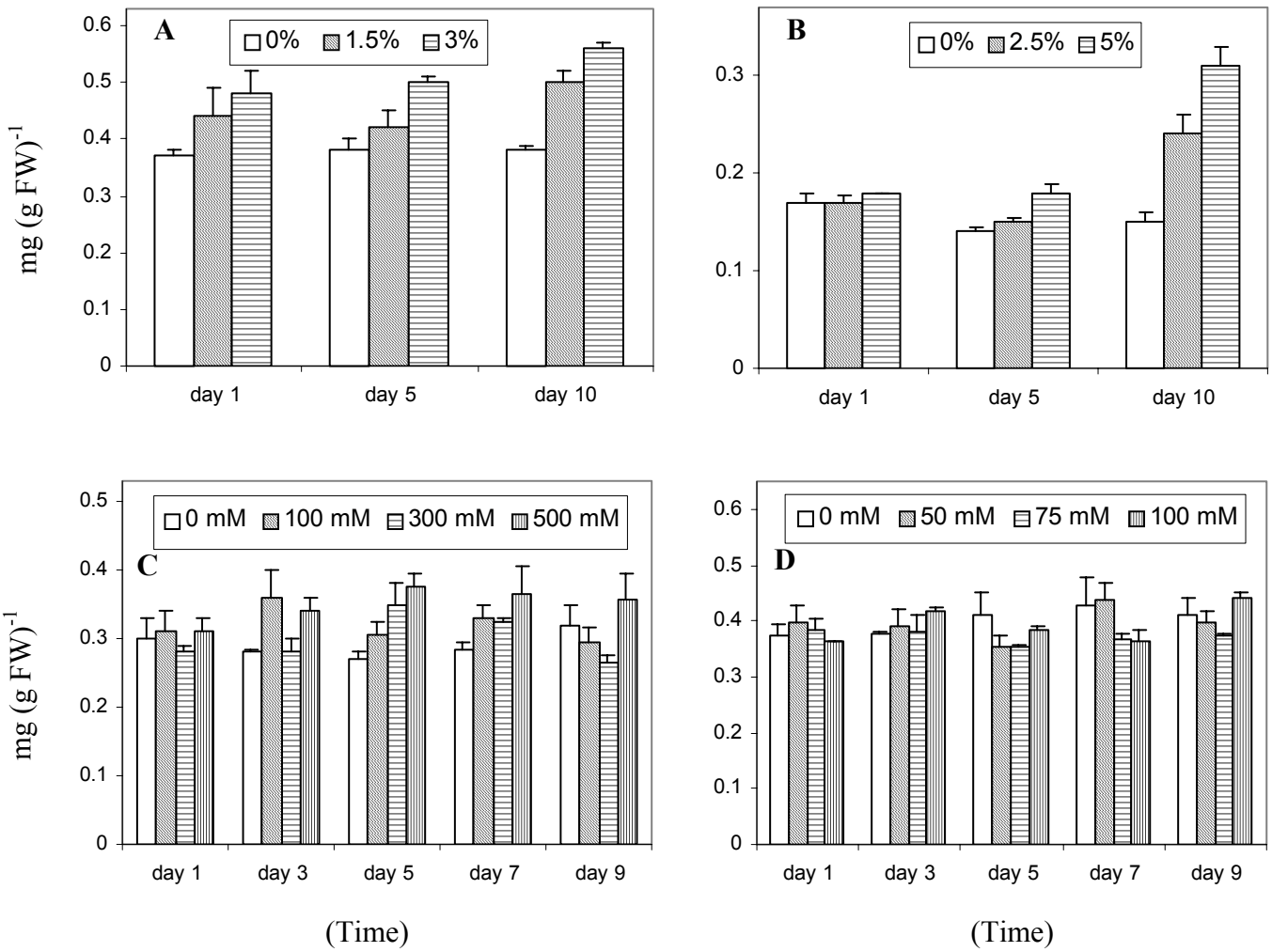


Fig. 3. Changes in the SQDG content in halophytes and glycophytes in response to salt treatment. (A) *Aster tripolium*, (B) *Sesuvium portulacastrum*, (C) *Thellungiella halophila*, and (D) *Arabidopsis thaliana* (ecotype Wassilewska). The graphs show the SQDG contents in the same plants shown in Figure 1. The plants were treated with the respective salt solutions for the days indicated.

4.2.4 Gas chromatography-mass spectrometry (GC-MS) analysis of fatty acids from sulfoquinovosyldiacylglycerol (SQDG)

Using the advances of GC coupled with MS the fatty acids profiles of SQDG from *Aster* and *Sesuvium* were analysed. First the method was developed and standardized as described in the Material and Methods part using the commercially available SQDG standard. It contains predominantly 16:0 (palmitic acid) and 18:3 (γ-linolenic acid), and trace amounts of 18:2 (linoleic acid) (Fig. 4).

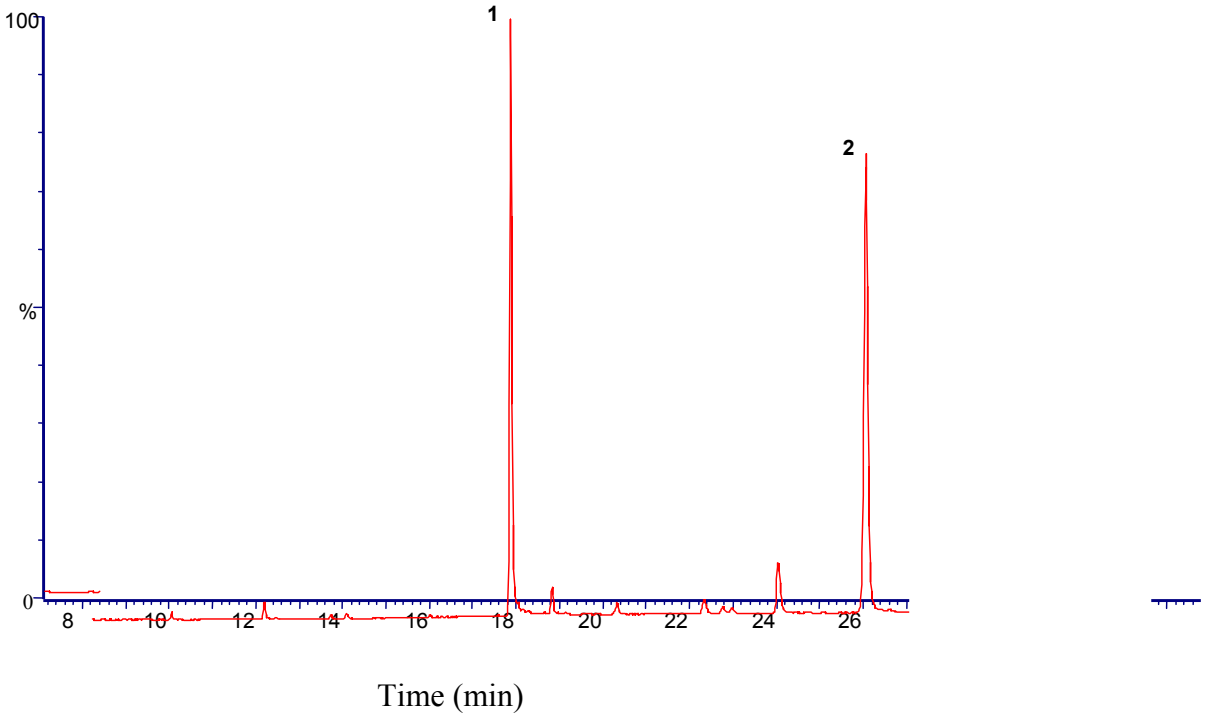


Fig. 4. Representative GC-MS total ion current chromatogram (TIC, corresponding to 100% of scale is indicated on the chromatogram) showing the composition of the commercially available SQDG standard (5 µg/µl). Peaks: 1, 16:0 (palmitic acid); 2, 18:3, (linolenic acid). Injection volume: 2 µl. SQDG was saponified by adding 0.5 ml of 0.5 M methanolic sodium hydroxide and heating to 80°C for 5 min, esterified with 1 ml of boron trifluoride methanol complex by re-heating for 5 min (80°C). After cooling and addition of 2 ml saturated sodium chloride solution, the fatty acid methyl esters (FAME) were extracted with 2 ml of n-hexane. Separation of FAME was achieved on a DB-WAX fused silica column (30 m × 0.32 mm i.d., film thickness 0.25 µm, J & W, Germany) using helium as carrier gas (3.2 ml min^{-1}) in Fisons 8060 gas chromatograph equipped with an on-column injector (100°C) directly coupled with a Fisons MD 800 mass spectrometer. The temperature of the ion source was 200°C, the electron energy for the electron impact mass spectra was 70 eV. (only the major peaks 1 and 2 were analysed in view with experimental importance)

Subsequently samples from *Aster* and *Sesuvium* grown for 10 and 7 days, respectively, with and without salt as described were analysed. The representative chromatograms show that the plant SQDG contain 16:0, 18:0 (stearic acid), 18:1 (oleic acid), 18:2, and 18:3 in various amounts and ratios (Fig. 5A-D). The fatty acid 16:1 (palmitoleic acid) was not detectable in any SQDG. To prove the reliability of the method for the determination of the fatty acid profiles the fatty acid composition of the SQDG from *Nicotiana tabacum* L. was determined and compared to published data (Bishop et al., 1985) which are in agreement with our data (data not shown). It is already visible from the chromatograms that the *Aster* plants treated with salt had changed fatty acid composition of their SQDG. Especially the 18:2

amounts were increased whereas the 16:0 percentage decreased (Fig. 5A and B). For *Sesuvium* smaller changes in the fatty acid composition could be observed (Fig. 5C and D). The results of the fatty acid composition of SQDG are summarized in table 2. In *Aster* the degree of unsaturation slightly decreased after one day of salt treatment in comparison to control plants because less 18:3 and more 18:2 species were detected. During longer salt treatment (10 days) the degree of unsaturation slightly increased because less 16:0 and almost double as many 18:2 species than in the controls were determined. In *Sesuvium* the degree of unsaturation increased after one day of salt treatment (more 16:0 species). As was already visible from the chlorophyll data the first day of high salt is challenge for the plant metabolism to induce stress defence mechanisms. After 7 days of salt treatment the 18:2 species clearly increased in comparison to the control in a similar way as in *Aster*. Although the SQDG species differ in their fatty acid composition their adsorption and distribution behaviour during the process of TLC was not changed as demonstrated by the identical retention times in the thin layer system used. The data show that although both halophytes *Aster* and *Sesuvium* contained higher SQDG contents when treated with NaCl, their fatty acid composition differed with respect to saturation.

Table 2 Fatty acids composition of SQDG in the leaves of *Aster tripolium* (top) and *Sesuvium portulacastrum* (bottom) watered with different concentrations of NaCl (*Aster*, 0%, 1.5%/257 mM, and 3%/513 mM; *Sesuvium*, 0%, 2.5%/428 mM, and 5%/856 mM NaCl) for several days. The composition is given in mol%. The values are the means of results obtained from three independent experiments.

Data from *Aster tripolium*

NaCl conc.	16:0	18:0	18:1	18:2	18:3
			day 1		
0%	32.4±6.6	10.1±1.9	11.0±4.0	17.1±6.1	29.8±5.1
1.5%	30.0±4.6	13.1±2.2	13.3±3.8	17.4±1.7	25.5±4.7
3%	32.6±7.8	8.3±1.7	9.8±2.2	25.5±7.3	23.4±3.9
			day 10		
0%	34.5±4.0	6.0±1.2	11.4±2.4	14.4±3.2	38.4±5.4
1.5%	34.9±4.7	5.2±2.5	9.8±0.7	25.2±2.4	24.9±4.1
3%	31.1±8.5	5.7±1.5	9.8±2.8	29.9±6.6	23.1±3.0

Data from *Sesuvium portulacastrum*

NaCl conc.	16:0	18:0	18:1	18:2	18:3
	day 1				
0%	33.1±1.1	6.1±0.5	20.4±0.05	25.7±1.8	15.1±2.9
2.5%	38.3±6.3	7.6±1.3	18.4±3.0	23.3±8.1	14.3±3.3
5%	39.8±5.7	6.4±0.5	15.9±6.6	25.3±6.6	12.3±2.3
	day 7				
0%	35.9±2.8	8.7±1.8	19.3±0.8	23.3±2.2	12.8±0.4
2.5%	37.8±4.1	7.5±2.5	18.6±1.7	21.7±4.4	12.4±1.7
5%	33.1±4.8	4.5±0.9	17.2±0.5	30.9±5.6	13.9±2.8

4.2.5 Liquid chromatography-mass spectrometry (LC-MS) of the sulfoquinovosyldiacylglycerol (SQDG) pool

We were not only interested in knowing the percentage of the different fatty acid classes in all SQDG molecules in the halophytic plants, but also wanted to elucidate the fatty acid composition at *sn*-1 and *sn*-2. For the commercially available SQDG standard molecular ions were registered at 812.8796 Da and 834.8245 Da corresponding to $C_{43}H_{73}O_{12}S$ and to $C_{43}H_{71}O_{12}S$ (data not shown). In the SQDG isolated from *Aster* ions were registered at 815.5008 Da and 837.4813 Da corresponding to elemental compositions of $C_{43}H_{75}O_{12}S$ (0.1 ppm) and of $C_{45}H_{73}O_{12}S$ (-0.2 ppm), respectively (Fig. 6A and 6B), among others (Fig. 6C). The molecular mass of 815.5008 Da corresponding to $C_{43}H_{75}O_{12}S$ is in good agreement with a 16:0/18:3 SQDG and the mass of 837.4813 Da ($C_{45}H_{73}O_{12}S$) matches the elemental composition of an 18:3/18:3 SQDG. Therefore SQDG from *Aster* contains also 18:3/18:3 together with other molecular species which was not reported previo

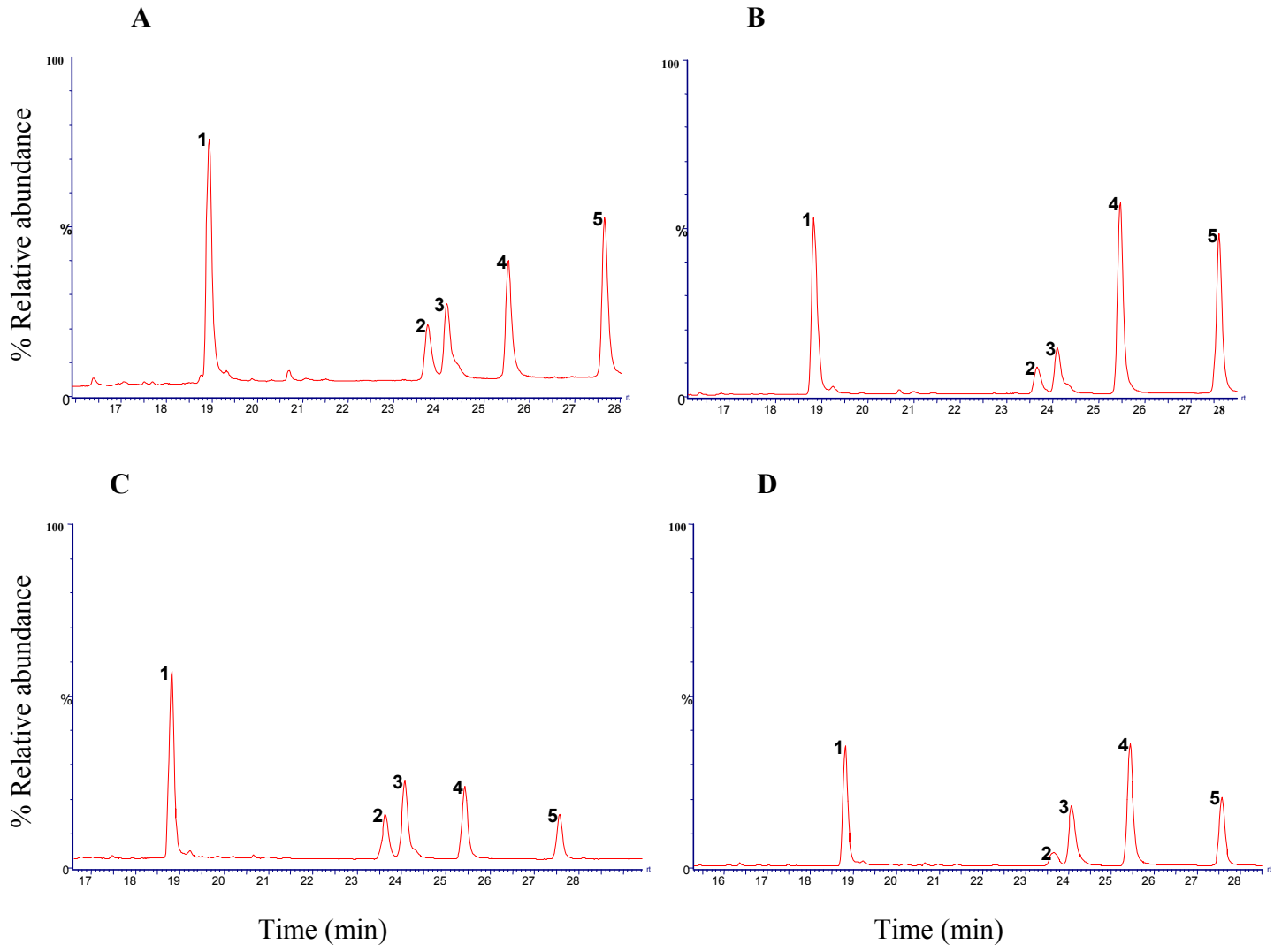


Fig. 5. Representative GC-MS total ion current chromatograms (TIC, corresponding to 100% of scale is indicated on the chromatograms) showing the composition of SQDG from (A) *Aster tripolium*, 0%, (B) *Aster*, 3%, (C) *Sesuvium portulacastrum* 0%, and (D) *Sesuvium*, 5%. The plant samples were harvested from the plants described in Figure 1 after 10 days and 7 days of the salt treatment, respectively. Peaks: 1, 16:0 (palmitic acid); 2, 18:0 (stearic acid); 3, 18:1 (oleic acid); 4, 18:2 (linoleic acid); 5, 18:3 (linolenic acid). Injection volume: 2 µl.

4.2.6 Physiological meaning of the results obtained

The halophytes *Aster* and *Sesuvium* both contain increased SQDG contents when treated with salt in comparison to control plants whereas the members from the Brassicaceae family *Thellungiella* and *Arabidopsis* do not show any significant differences in their SQDG contents with respect to the salt treatment. Up to now the SQDG contents and their fatty acid composition were predominantly analysed in glycophytes, like spinach, tobacco, wheat, and cucumber (Bishop et al., 1985). SQDG in alfalfa leaves showed almost equal quantities of

16:0 and 18:3 fatty acids whereas the SQDG from *Chlorella pyrenoidosa* contained 16:0; 18:1, and 18:3 fatty acids (O'Brien and Benson, 1964). In *Nerium oleander* L. the major molecular species in SQDG were 18:3/16:0 (40.2%), 18:1/16:0 (18.3%), 16:0/16:0 (11.8%) (Orr and Raison, 1987). The positional distribution of fatty acids on *sn*-1 and *sn*-2 was investigated by Bishop et al. (1985). All samples of SQDG isolated from different the species, such as spinach, tobacco, wheat, and cucumber contained higher contents of 16:0 than the corresponding MGDG and DGDG. The positional distribution of 16:0 demonstrated that in the 16:3-plants significant amounts of these acids are present at both the *sn*-1 and *sn*-2 positions, demonstrating that both prokaryotic and eukaryotic mechanisms for DAG production contribute to the biosynthesis of SQDG. In the 18:3-plants 16:0 acids are predominantly located in the *sn*-1 position of SQDG, again demonstrating the origin of the DAG as being the eukaryotic pathway. Unfortunately, it is not known to which group of plants *Aster* and *Sesuvium* belong to.

The results obtained so far with respect to changes in SQDG contents due to environmental conditions are rare. It has been reported that in autumn there was an increase in SQDG contents in *Pinus silvestris* (Oquist, 1982). The effect of low temperature on the lipid content and composition of the thylakoid membrane was investigated in *Pisum sativum*. Both the relative amounts of lipid classes and the degree of saturation were not greatly changed during growth at cold and warm conditions. However, it was demonstrated that in cold-grown plants there were slightly higher 18:3 and lower 18:2 contents for all glycolipids (MGDG, DGDG, and SQDG). Interestingly, a chloroplast envelope fraction of cold grown plants contained a higher level of fatty acid unsaturation (Chapman et al., 1983). It was concluded that SQDG has no role in chilling sensitivity because it lacks sufficient quantities of di-16:0 molecular species (Murata and Hoshi, 1984).

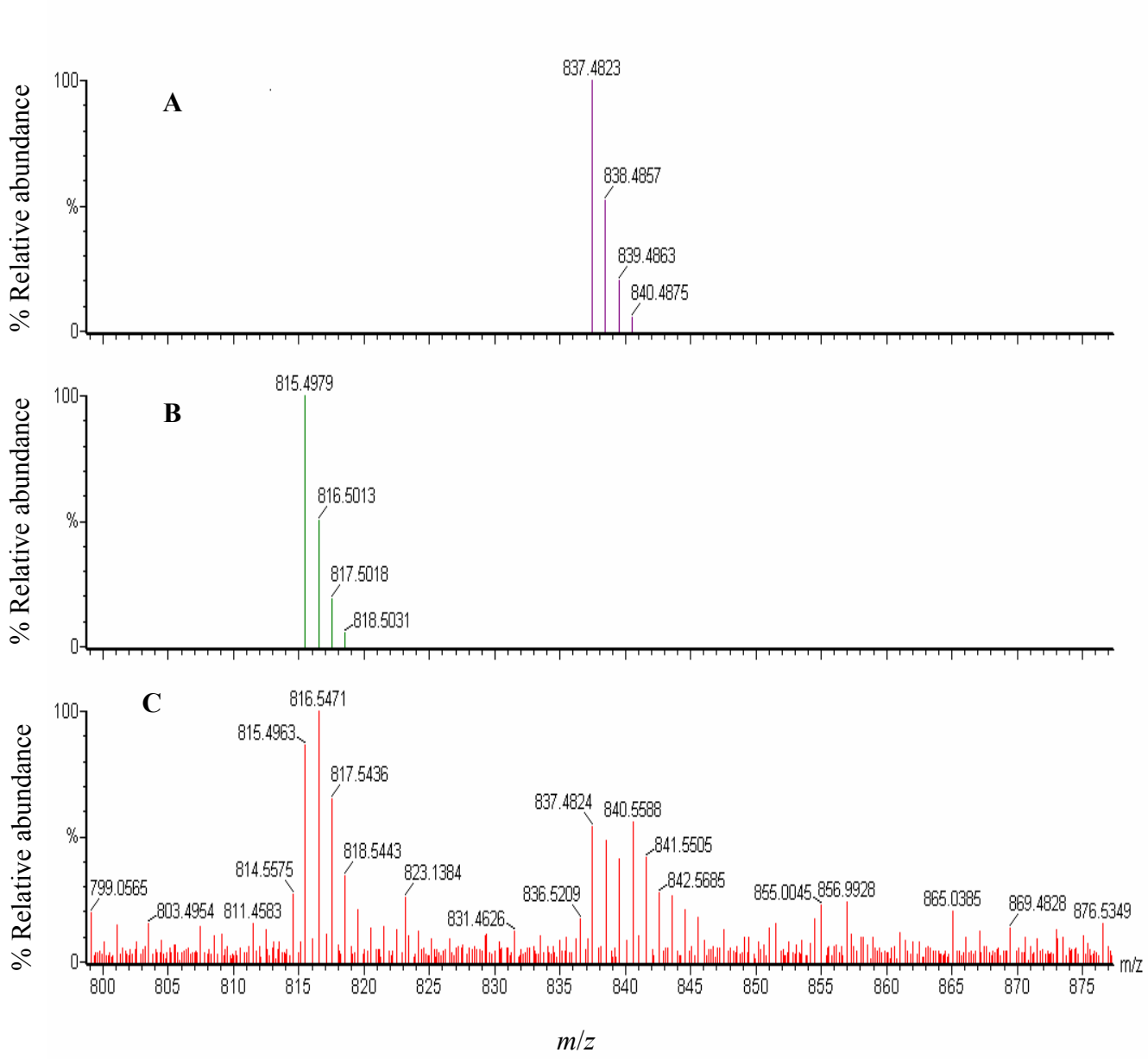


Fig. 6. LC-MS spectra of SQDG extracted from *Aster tripolium* showing two different molecular ion peaks at (A) 837 Da, (B) 815 Da, and (C) in the complete mass spectrum with 815 and 837 Da peaks. Single mass analysis with 0.1 and –2.0 ppm tolerance for 815 and 837 Da, respectively. Mass spectra were recorded in the negative electrospray mode (cone voltage 30 V) by an LCT time of flight mass analyser (Micromass, Manchester, UK), equipped with a LockSpray™ unit for exact mass measurement.

With respect to 18:3-plants the conclusion was supported by the data from Bishop et al. (1985), however, it remains possible that the di-16:3 molecular species of SQDG could be synthesized in some 16:3-plants in which chloroplast- derived DAG is utilized. Lines of *Zea mays* which differed in their sensitivity to chilling were analysed for their high-temperature melting fraction of SQDG. However, the differences determined could not be related to their chilling sensitivity (Kaniuga et al., 1999). In our experiments any temperature effects can be excluded because of the almost constant temperature in the green house. Therefore we assume

direct effects of the salt treatment on the SQDG contents. In wheat cultivars, it was reported that a drought-tolerant line has increased SQDG contents during water stress but SQDG contents decreased in the drought-sensitive line during the same conditions (Quartacci et al., 1995). When NaCl was given to the external medium of seedlings of the glycophyte *Hordeum vulgare* L., it has been reported to have unchanged SQDG content compared to the control (Muller and Santarius, 1978). In salt-sensitive lines of *Beta vulgaris* L. the sulfolipid contents remained unchanged due to NaCl in the external medium but in salt-tolerant lines they have reported increased sulfolipid content (Kuiper and Kylin, 1981; Stuiver et al., 1981). We have grown the *Arabidopsis* mutant *SQD2* mutant with salt concentrations up to 100 mM and observed reduced growth in comparison to wild-type plants treated in the same way of about 50% (data not shown). Therefore the *SQD2* seems to be less salt-tolerant which might indicate a role for sulfolipids in salt tolerance even in glycophytes.

It was hypothesized a long time ago that SQDG are involved in stabilizing ATPase complexes and PSII (Pick et al., 1985). Recently more evidence emerged that the role of SQDG is linked to proper functioning of PSII (Minoda et al., 2002, 2003). The authors demonstrated that a *Chlamydomonas reinhardtii* mutant deficient in SQDG biosynthesis showed almost 40% reduction in PSII activity and could be restored back when supplemented with SQDG and at the same time chemical modifications of SQDG impaired the activity of PSII with the conclusion that SQDG contributes to maintain the conformation and efficiency of PSII complexes in *Chlamydomonas*. Similar effects were reported for a *Chlamydomonas SQD1* mutant well characterized on the genetical level that showed increased sensitivity to a diuron herbicide, a herbicide binding to the Q_B receptor site of PSII, and thereby blocking PSII activity, suggesting a role for SQDG in photosynthesis. Alterations in PSII in the mutant might make it more accessible for the herbicide or less stable under the experimental growth conditions (Riekhof et al., 2003). The mutant had also reduced growth under phosphate limitation whereas SQDG deficiency does not seem to have an adverse effect on growth under optimal growth conditions. SQDG might also be critical for photosynthesis in the cyanobacterium *Synechocystis* sp. PCC6803 because previous attempts to disrupt a gene involved in SQDG biosynthesis failed in this particular organism without exogenous supplementation of SQDG (Güler et al., 2000). This has to be confirmed still in higher plants, e.g. by performing herbicide experiments with the *SQD1* and *SQD2 Arabidopsis* mutant.

The nutritional status of the plants influences the SQDG content as was demonstrated for some Brassicaceae plants. Sulfur deprivation has a decisive impact on lipid metabolism in *Brassica oleracea* (DeKok et al., 1997). There was a substantial decrease in the levels of

SQDG in both roots and shoots when expressed in a total lipid basis. However, when expressed on a chlorophyll basis the SQDG contents remained constant indicating a strong affiliation of SQDG and chlorophyll. In *Aster* there was also a positive correlation between chlorophyll and SQDG contents with increasing NaCl in the watering solution. Trosper and Sauer (1968) made the suggestion that SQDG might assist in the orientation of chlorophyll molecules in the membranes. It would be interesting to follow this idea. However, the *Arabidopsis* mutant *SQD2* the SQDG contents are reduced to almost undetectable levels whereas the chlorophyll contents were unchanged in comparison to wild-type (Yu et al., 2002).

A major role of SQDG in glycophytes might be the exchange of phospholipids to SQDG during phosphate starvation. Under normal growth conditions about 30% of P_i is bound in phospholipids which might be released under phosphate deficiency when increasing amounts of SQDG take over the role of phopholipids at least in *Arabidopsis* (Essigmann et al., 1998). To prove this physiological function of SQDG for halophytes similar experiments could be done with a parallel application of phosphate deficiency and salt stress.

The increasing amounts of SQDG during salt treatment might also play a role in signalling processes because it was shown that spinach annexin binds in the presence of 100 µM Ca^{2+} not only to envelope membranes of purified chloroplasts but also to vesicles consisting of phosphatidylcholine (PC) and SQDG (Seigneurin-Berny et al., 2000). However, only SQDG and not PC was accessible in the asymmetric outer envelope membrane and might communicate any signals via annexin to the cytoplasm or vice versa. In some halophytes SQDG might play a role to protect the plant against salt stress. To follow this hypothesis we need to construct and analyse halophytic mutants deficient in SQDG biosynthesis and then investigate its conditional role in salt tolerance. *Thellungiella* might have developed different strategies with respect to salt tolerance. It would be very interesting to start a lipomics project to investigate the abundance of all lipids forming the different membranes of the cell and to determine their fatty acid composition during different environmental conditions especially of high salt.

5 Connecting sulfur metabolism and salt tolerance in halophytes

5.1 INTRODUCTION

Crop production is affected by numerous environmental factors, with soil salinity and drought having the most detrimental effects. Attempts to improve yield under stress conditions by plant breeding have been unsuccessful, primarily due to the multigenic origin of the adaptive responses. This has led to research into salt tolerance using halophytic plants with the aim of using them as crop plants. Stress management is important for halophytes as well as glycophytes to survive extreme environmental conditions. This could be heat, cold, drought, higher concentrations of ions, heavy metals or shortage of growth-related elements such as potassium or phosphate. All plant stress effects inhibit growth, and thus plants are hampered by high light conditions, since the light can not be used for sufficient growth. This leads to decreased CO_2-fixation rates and thus to the probability of oxygen radical formation increases since the electron transport energy is not used up for CO_2-fixation. Thus defence mechanisms against oxygen radical damages are necessary. This is partly overcome by oxygen radical detoxification by the glutathione-ascorbic acid system where glutathione is necessary for the reduction of ascorbic acid in the H_2O_2-cycle (Halliwell and Gutteridge, 1989). For enhanced glutathione formation an increase of the sulfate reduction pathway of the plant is needed. Furthermore, plants will have to synthesize organic molecules as radical scavengers, such as trehalose. These metabolic adaptations need energy as well. For these biosynthetic routes the plant will need high ATP-formation, which is dependent on proper membrane functions of the chloroplasts. Sulfolipids occur in the membrane of plastids and have been shown to be needed for ATP-formation within the chloroplast membrane and for the stabilization of the photosystems (Krupa and Bazynski, 1977; Pick et al., 1985).

High salinity is a special case of plant stress. High concentrations of NaCl force the plant to cope with effects of osmotic tolerance in general and the problem of NaCl toxicity within the plant cell as specific problem. Many enzymes are inhibited by higher NaCl concentrations, thus these enzymes should be changed to NaCl tolerant types at higher concentrations of NaCl in the cell or these concentrations of NaCl have to be avoided (Zhang et al., 2001). Whereas general phenomena of salt tolerance have been documented during the last 50 years (Staples and Toenniessen, 1984), our understanding of the key metabolic functions inhibited by NaCl within the plant cell are still emerging. It is accepted that there is no single specific point for salt tolerance. Therefore we need to screen important pathways for their impact on salt stress and salt tolerance.

Since sulfur is a key element for the plant stress management and for plant energy production the effects of salt stress on sulfur metabolism in salt tolerant plants was analysed. We would like to investigate the role of sulfur-containing compounds as well as the respective biosynthetic enzymes in salt stress of halophytes and glycophytes. Two enzymes in sulfur metabolism were selected for detailed analysis. Whereas *O*-acetyl-L-serine(thiol)lyase (OAS-TL) catalyses the formation of L-cysteine, ß-cyanoalanine synthase (CAS) plays a crucial role in catalysing the conversion of cyanide and cysteine to ß-cyanoalanine (Maruyama et al., 2000). Therefore both enzymes are involved in controlling the size of the cysteine pool, the key molecule providing reduced sulfur for a number of biosynthetic processes in plants. The halophytic plants *Aster tripolium* and *Sesuvium portulacastrum* were chosen for the detailed analysis because both of them were shown to have the potential as future crop plants. The exact role of sulfolipids in salt tolerance mechanisms is still not known. The sulfolipid sulfoquinovosyl diacylglycerol is an abundant sulfur-containing glycerolipid that is preferentially associated with photosynthetic membranes of higher plants and other photosynthetic organisms. The final biosynthetic steps take place in the chloroplasts of higher plants (Benning, 1998). It was shown that the SQD1 protein catalyzes in *Arabidopsis* the formation of UDP-sulfoquinovose from UDP-glucose and sulfite, derived from the sulfate reduction pathway leading to cysteine in the chloroplast (Sanda et al., 2001). It was suggested that sulfolipids play a role in stabilizing the photosystems and the ATPase complex (Pick et al., 1985) and may be important under phosphate-limiting conditions (Essigmann et al., 1998). We would like to clarify the putative role of sulfolipids in salt tolerance mechanisms of halophytes and glycophytes as well as the influence of salt stress on certain aspects of sulfur metabolism.

5.2 RESULTS

5.2.1 Description of the phenotype and conductivity measurements

The growth rate and therefore the plant size of both species were decreased when treated with increasing NaCl concentrations in the watering solution. The phenotype of *Aster tripolium* plants grown under the same conditions has been described previously in more detail (Lieth et al., 2002). The leaves of *Aster* plants treated with NaCl showed wilting symptoms occurring after 10 d of watering. However, the plants still grew and recovered easily when watered without NaCl in the watering solution. *Sesuvium* plants showed the effects of salt uptake faster; the leaves does not show any wilting symptoms. One has to keep

in mind that the salt concentrations given were not the end concentrations rather the amount of NaCl in the watering medium, which implies it is a gradient increase in NaCl and at the end of the experiment the concentration is much higher and also different concentrations in *Aster* and *Sesuvium* because pre-tests have shown that *Sesuvium* plants have higher salt tolerance levels than *Aster* plants (data not shown). Surprisingly, *Sesuvium* plants lost their succulent morphology within a week of salt treatment. To demonstrate that both experimental plants took salt up from the growth medium the conductivity was determined in soil without any plant, soil with growing *Aster tripolium* and *Sesuvium portulacastrum* plants treated with 3% NaCl in the watering solution (Fig. 1). The results indicate that in both cases the conductivity of the soil containing plants was lower than the control soil without plants. From these results we can conclude that our experimental plants *Aster* and *Sesuvium* both took salt actively up.

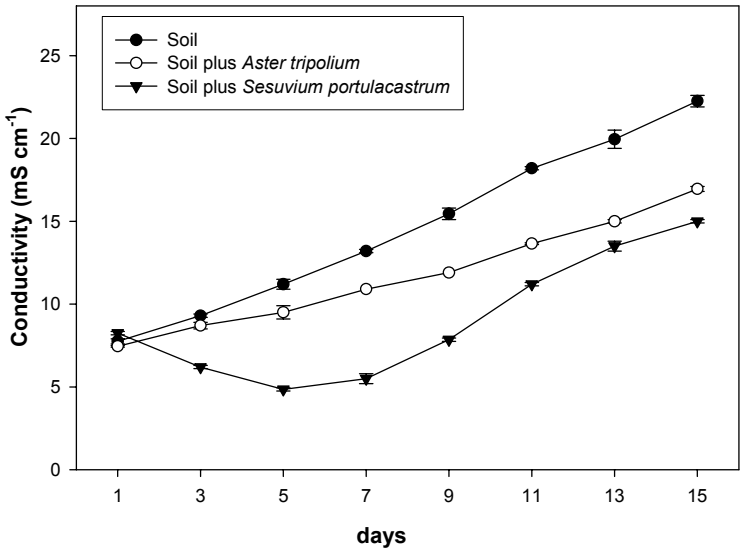


Fig. 1. The graph shows the conductivity values of soil without any plant, soil with growing *Aster tripolium* plants and with *Sesuvium portulacastrum* plants grown under 3% NaCl in the watering solution. Soil samples were taken for 15 days every second day starting from day 1.

5.2.2 Sulfolipid contents of *Aster tripolium*

Aster plants were treated with different NaCl concentrations in the watering solution (0, 1.5, and 3%) for 1 to 9 days. The leaves and roots were separated, frozen in liquid nitrogen, and analysed for their sulfolipid contents. The sulfolipid contents were always enhanced in *Aster* leaves when watered with high salt concentrations over the complete experimental period in comparison to plants watered without any additional NaCl in the watering solution (0% in comparison to 1.5 and 3%, Fig. 2A). The largest differences were observed at day 7 of

the treatment. Afterwards the sulfolipid contents decreased slightly indicating a role in short-term adaptation. In the long-term adaptation to salt stress additional mechanisms might play a role. In roots of the same *Aster* plants the sulfolipid contents are about 5x lower than in *Aster* leaves, probably because of the lower abundance of plastids in the non-photosynthetic tissue than chloroplasts in the green tissue. Overall the sulfolipid contents are not increased in roots of salt treated plants (Fig. 2B). In addition to the determination of mg sulfolipid per gram fresh weight the amount of sulfolipid per gram dry weight was determined. The absolute values were about ten times higher, however, the ratios between the different treatments remained the same (data not shown).

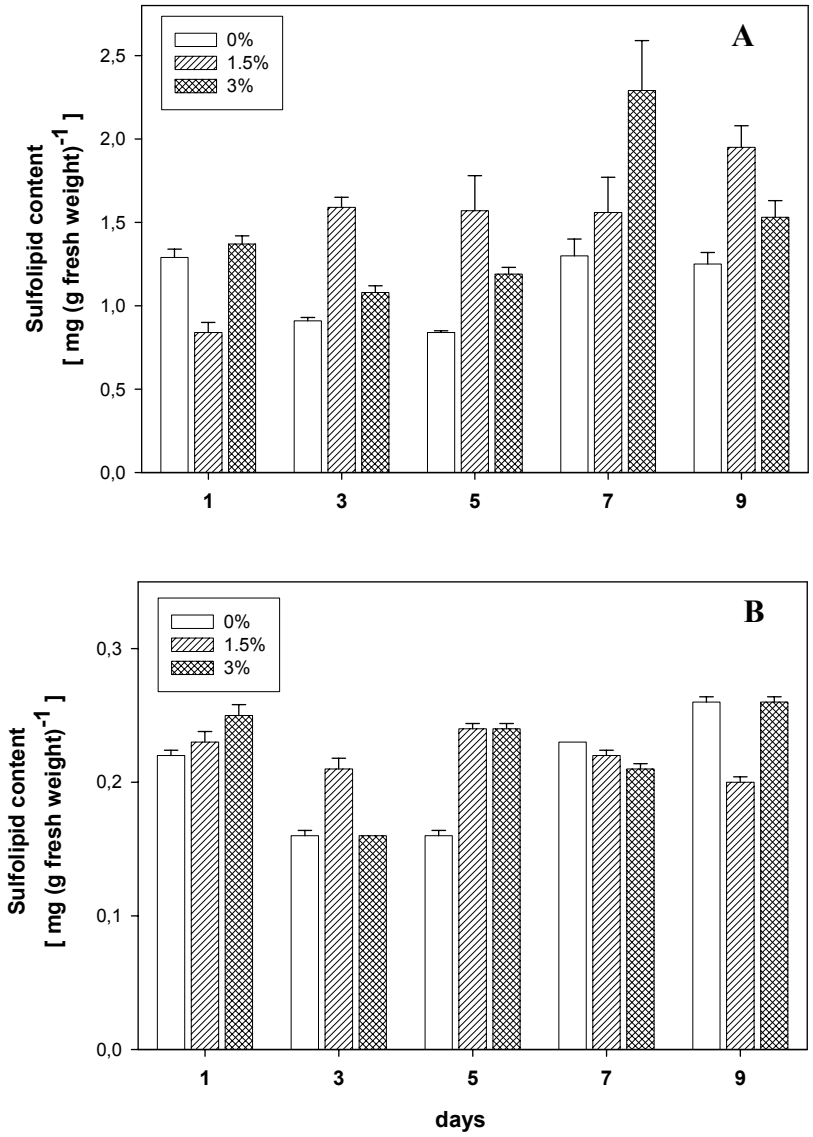


Fig. 2. The graph shows the sulfolipid contents in *Aster tripolium* leaves (A) and roots (B) grown under 0, 1.5, and 3% NaCl in the watering solution and harvested in the order of day 1 to 9. Standard errors are given.

5.2.3 Sulfolipid contents of *Sesuvium portulacastrum*

Sesuvium cuttings were treated with different NaCl concentrations in the watering solution (0, 2.5, and 5%) for 1 to 7 days. The leaves were harvested after the respective days and the sulfolipid contents determined as described. The sulfolipid contents were slightly enhanced in *Sesuvium* leaves of plants watered with high salt concentrations over the complete experimental period in comparison to plants watered without any additional NaCl in the watering solution (0% in comparison to 2.5 and 5%, Fig. 3). The differences remained almost constant during the treatment.

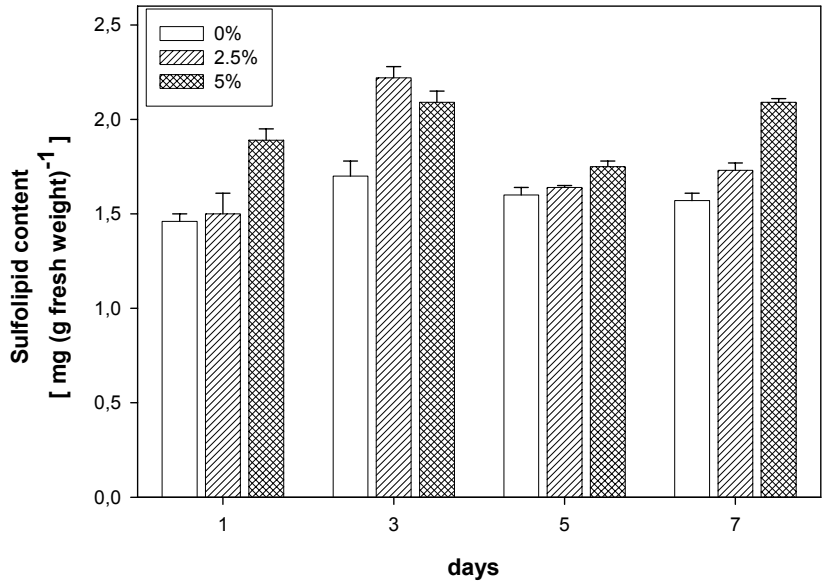


Fig. 3. The graph shows the sulfolipid contents in *Sesuvium portulacastrum* leaves of plants grown under 0, 2.5, and 5% NaCl in the watering solution and harvested in the order of day 1 to 7.

5.2.4 Enzyme activities

The enzymatic activities of OAS-TL and CAS were determined in leaves and roots of *Aster* plants treated for 2 or 10 days with 0, 1.5 or 3% NaCl in the watering solutions. The activities of OAS-TL, the final enzyme for incorporation of sulfide into cysteine, were severely inhibited with more than 40% reduction during prolonged growth on high salinity in comparison to the control plants (Fig. 4A and B). The decrease can be observed in leaves (Fig. 4A) and roots (Fig. 4B) to the same extent. The CAS activity in the same plant material showed a decrease in the enzyme activity of more than 50% in leaves (Fig. 4C). In the roots of the same *Aster* plants the activity was reduced to less than 30% from day 2 to day 10 (Fig. 4D).

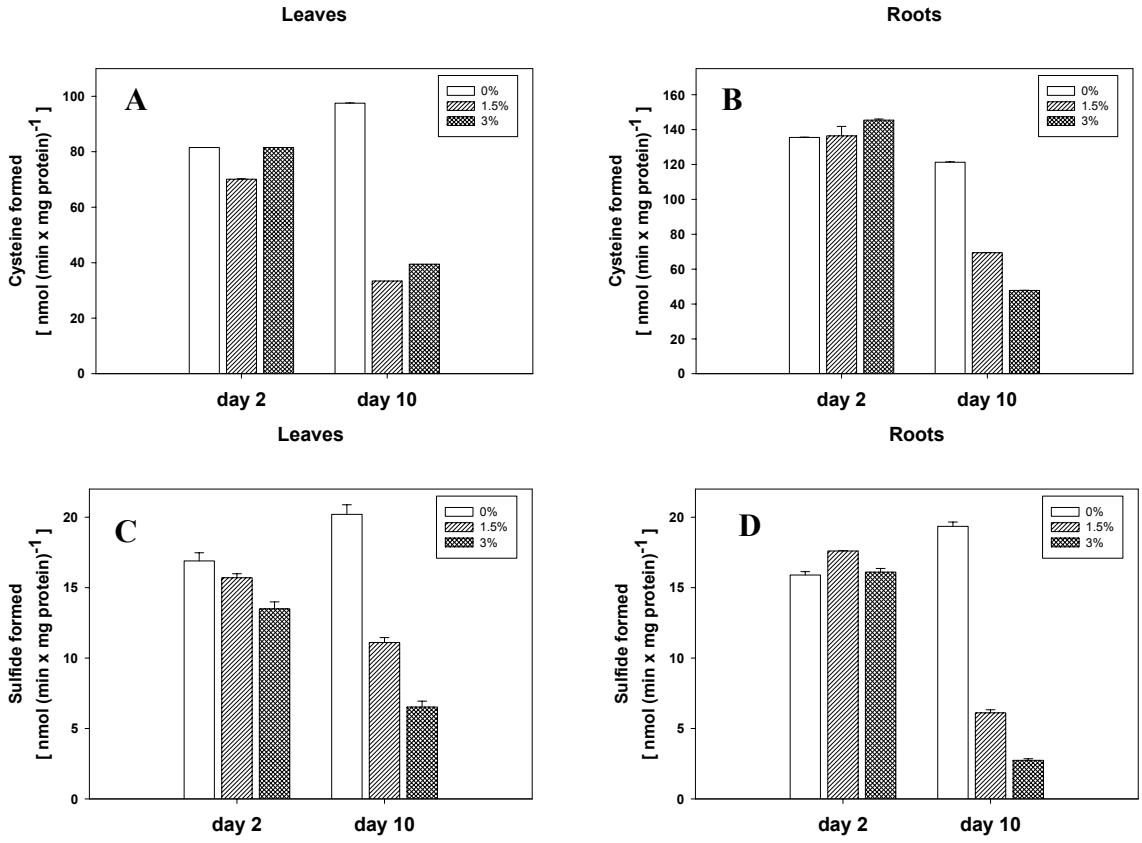


Fig. 4. Determination of enzyme activities in salt treated plants. The upper part of the graph shows the OAS-TL (*O*-acetyl-L-serine(thiol)lyase) enzyme activity in *Aster tripolium* leaves (A) and roots (B) grown under 0, 1.5, and 3% NaCl in the watering solution and harvested during day 2 and 10. The product formation of L-cysteine was determined. The lower part of the graph shows the CAS (ß-cyanoalanine synthase) enzyme activity in *Aster tripolium* leaves (C) and roots (D) grown under 0, 1.5, and 3% NaCl in the watering solution and harvested during day 2 and 10. The product formation of sulfide was measured.

5.2.5 Western blot analysis

Total protein extracts from the same plants used for the enzyme activity measurements were separated by SDS-PAGE and blotted onto membranes. Antibodies specific for OAS-TL and CAS proteins (Hatzfeld et al., 2000) were used for immunodetection. The protein pattern of control and salt treated plants look very similar demonstrated by the Coomassie-stained gel of the total protein extracts (Fig. 5). It can be seen that two OAS-TL proteins are detected by the blotting technique. These two proteins can be found in leaves and in roots as well. The levels of larger of both CAS isoformes were also slightly reduced by salt treatment in leaves.

This isoform was not detectable in roots. The total amount of protein used for blotting was normalized, however, it can be seen that especially in the roots the bands for the OAS-TL are

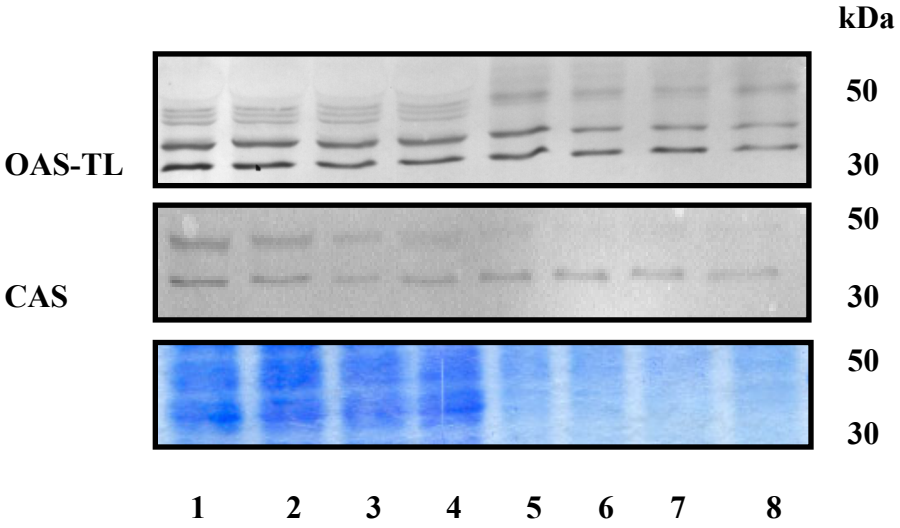


Fig. 5. Western blot of OAS-TL and CAS proteins using antibodies raised against spinach OAS-TL and CAS. The plants were harvested as indicated, total protein extracts were prepared and separated by SDS-PAGE as described in the materials and methods part. The lanes were loaded according to the following scheme: lane 1: leaves without NaCl, day 2; lane 2: leaves with 3% NaCl, day 2; lane 3: leaves without NaCl, day 10; lane 4: leaves with 3% NaCl, day 10; lane 5: roots without NaCl, day 2; lane 6: roots with 3% NaCl, day 2; lane 7: roots with 0% NaCl, day 10; lane 8: roots with 3% NaCl, day 10. The Coomassie-stained gel of the total protein extracts is shown as control at the bottom at the figure.

clearly weaker stained than the control without salt treatment, confirming the data obtained by enzymatic measurement. Therefore we can exclude a general down regulation of the metabolism. This clearly shows that enzymes involved in either cysteine formation as shown for the OAS-TL or for further metabolism of cysteine to ß-cyanoalanine are targets of salinity imposed on the regulation of sulfur metabolism.

5.3 DISCUSSION

We have chosen to analyse effects of salt stress on sulfur metabolism in salt tolerant plants, since sulfur metabolism is a key element for the plant stress management and for plant energy production as well. Sulfate availability in freshwater ecosystems is around 0.12 mM, whereas sea water has a sulfate concentration of 28.8 mM, which is about 200-fold higher (Oren, 2002). Therefore sulfate limitation should not be expected in salt-tolerant plants. There is, however, the possibility that costal marsh lands are partly anaerob leading to higher sulfide

concentrations, especially when sulfate concentrations are high. Sulfide in higher concentrations is toxic to plants (Oren et al., 1979) and thus special avoiding strategies against higher sulfide concentrations in roots might be beneficial or even necessary for salt tolerance of plants.

The plants *Aster tripolium* and *Sesuvium portulacastrum* were used for our studies, since these are salt tolerant plants which can be grown at high salt concentrations conveniently in the green house with good production of biomass. This eases biochemical and molecular investigations. We have chosen to analyse a) sulfolipid changes in these two plants and b) to monitor possible changes in the cysteine-forming capacities as the last step of assimilatory sulfate reduction for the formation of L-cysteine on the one hand and cysteine-degrading capacities on the other hand.

That the plants do take up NaCl can be clearly seen in our experiments by the difference of the conductivity of the soil alone compared to the conductivity of soil and plants. Plants were affected by prolonged NaCl-treatment leading wilting symptoms. Whereas the response of *Aster tripolium* was slowly building up with a maximum effect after 10 days, *Sesuvium portulacastrum* took up NaCl faster and the loss of succulence occurred earlier peaking within 7 days.

The sulfolipid content of *Aster tripolium* leaves was always higher in salt-treated plants compared to the control without NaCl addition (see Fig. 2A) and the differences were almost constant during the growth period whereas the sulfolipid contents in the roots increased only slightly (Fig. 2B). The sulfolipid contents in *Sesuvium portulacastrum* were always slightly higher in the leaves when compared to the non-treated plants (Fig. 3). It has been shown previously that in salt-tolerant lines of sugar beet (*Beta vulgaris*) the sulfolipid contents increased when watered with NaCl-containing solutions (Stuiver *et al*. 1981). This might indicate that higher sulfolipid concentrations could be beneficial for coping with salt stress especially by optimising ATP-formation to secure higher energy demand. However, more data are needed for a generalization of these observations.

Our experiments, however, clearly showed that enzyme(s) of the assimilatory sulfate reduction sequence were severely affected by higher salt concentrations. This is evident for the OAS-TL activity for *Aster tripolium,* where an effect was already seen two days after treatment. A severe decrease of enzyme activity was monitored after 10 days of growth, and this was true for leaves and for roots. The same results were observed for the CAS activity in these plants. The effects were even more pronounced when comparing the control and treated plants after 10 days of growth with a decline of over 30% of the enzymatic activities. Blotting

of total protein extracts from leaves and roots confirmed these data, however, the effect in the roots was more dramatic compared to the effects observed in the leaves. These data show that there is a clear effect of higher salt concentrations on one key enzyme of sulfate assimilation, suggesting that sulfur-related effects are involved in salt tolerance and that sulfur metabolism is one possible important key for understanding salt stress. One has to keep all the experiments were done using sea salt so the sulfide concentrations even in control might be different when using NaCl in watering medium.

It was shown previously by Barroso et al., (1999) that OAS-TL proteins are one possible target of salt tolerance in glycophytes. It was demonstrated that in *Arabidopsis thaliana* the expression of the cytosolic OAS-TL was significantly induced by exposure to salt stress. Addition of NaCl to mature plants induced a rapid accumulation of the mRNA throughout the leaf lamina and roots, and later on in stems, being mainly restricted to vascular tissues. The results suggest that a high rate of cysteine biosynthesis is required in *Arabidopsis* under salt stress necessary for a plant protection or adaptation mechanism. This hypothesis was supported by the observation that intracellular levels of cysteine and glutathione increased up to 3-fold after salt treatment. However, recently it was shown that *Brassica napus* plants showed a 3-fold increase in cysteine and glutathione contents in wild-type plants exposed to salt stress whereas in overexpressing the vacuolar Na^+/H^+ antiporter the levels of both compounds did not increase in response to salt stress (Ruiz and Blumwald, 2002). The induction of cysteine and glutathione synthesis during salt stress in the wild-type plants suggests a possible protective mechanism against salt-induced oxidative damage. On the other hand, the low levels of cysteine and glutathione in salt-tolerant transgenic plants confirm the role of vacuolar Na^+ accumulation and ion homeostasis in salt tolerance. To understand the role of both sulfur compounds it would be important to determine their levels in salt-treated *Aster* and *Sesuvium* plants. Effects of sulfur metabolism on salt stress have been documented also for an ATP-sulfurylase from *Arabidopsis thaliana*. 84 genes have been identified so far, which are enhanced under salt stress conditions using expression profiles by northern blot analysis (Gong et al., 2001).

A further link of sulfur metabolism and drought stress, and thus salt stress as well, can be seen by the recent discovery that functional abscisic acid biosynthesis is necessary for drought-related regulation. Map-based cloning has shown that the gene *Aba3* encodes a molybdenum cofactor (MoCo) sulfurase. MoCo sulfurase catalyses the generation of the sulfurylated form of MoCo, a cofactor required by aldehyde oxidase that functions in the last

step of abscisic acid biosynthesis in plants (Bittner et al., 2001; Xiong et al., 2001). This again shows a very intrinsic network of sulfur metabolism and drought or salinity stress in plants.

A shortage of reduced sulfur availability in the form of cysteine, as a consequence of decreased activities of OAS-TL, will definitely also lead to a shortage of iron-sulfur clusters which are needed for many proteins of the energy-handling systems, e.g. ferredoxins and aconitase (Walden, 2002). Thus iron metabolism and as a consequence of that the correct control of energy metabolism may be affected by salt stress as well. It was shown very recently by a study of salt tolerance mechanisms in *Bacillus subtilis* that high salt concentrations interfere with iron metabolism leading to iron limitation in these bacteria (Hoffmann et al., 2002). Since the enzyme APS-reductase involved in the reduction of sulfate to sulfite is an iron-sulfur protein as well (Kopriva et al., 2001) there might be a close connection between sulfate assimilation and iron availability in higher plants. Iron availability can be limiting for growth of bacteria (Hantke, 2001) and plants even under non-saline conditions (Curie et al., 2001), thus iron chelators are important for iron aquisition. Salt-treated soils may, however, be more critical not only for micro-organisms but also for higher plants when these soils become anaerobic and iron is fixed as FeS due to dissimilatory sulfate reducers. This is far more critical using sea water since the sulfate content in seawater is about 200-fold higher compared to fresh-water ecosystems (Oren, 2002).

Finally we would like to gain basic knowledge about salt tolerance phenomena. For detailed studies on the molecular level cDNA libraries from halophytic plants are necessary to be able to isolate genes involved in salt tolerance in general. This is particularly important for genes affected by salt stress as discussed for sulfolipid biosynthesis and cysteine formation which could serve as molecular markers for species and varieties differing in their salt tolerance. Therefore initial studies have been done to construct cDNA libraries of halophytic species that will enable us to use molecular genetic tools for further detailed studies.

6 Role of plasma membrane ATPase in salt tolerance

6.1 INTRODUCTION

Halophytes are plants that have been naturally adapted to and selected by saline environments and are distinguishable from glycophytes by their capacity to cope with excessive levels of ions with high proficiency. Although at low salinities NaCl accumulation in halophytes are often higher than those in glycophytes, uptake is not proportional to external salinity, and is curtailed at higher salinities (Greenway and Munns, 1980; Flowers et al., 1986). The sea aster *Aster tripolium* L. grows in the upper salt marsh and tolerates coastal seawater salinity. Seawater contains beside a sodium concentration of 3.5% an additional multi-ion cocktail. Ions such as Na^+, Ca^{2+}, Ba^{2+} or Cs^+, that are present in seawater from mM to μM range can block several ion transport processes or cause stress by water deprivation (Uozumi et al., 1995). One important strategy is to adjust the osmolarity to make sufficient water available for growth and development (Apse and Blumwald, 2002). This can be enhanced by K^+ accumulation, synthesis of osmolytes as well as compartmentalization of toxic ions in the cell vacuole and in the apoplast (Zhu et al., 1998; Su et al., 2001; Munns, 2002). Since the plasma membrane (PM) is the first cellular barrier for small molecules, many key processes involved in sensing and adaptation to alternating or extreme salt conditions are PM-localized (Blumwald et al., 2000; Serrano and Rodriguez-Navarro, 2001).

The vital functions of PM are fulfilled by a population of proteins which does not exceed 500 polypeptides for the model plant *Arabidopsis thaliana* and that can be estimated to approximately 750 polypeptides in the case of species with larger and more complex genomes such as tobacco (Masson and Rossignol, 1995). However, little information is available concerning PM proteins (Sussman, 1994), and only recently proteomic data of PM proteins became more abundant (Santoni et al., 1999; Kawamura and Uemura, 2003).

A recent breakthrough in improving salt tolerance in glycophytes was obtained producing transgenic tomato plants expressing a vacuolar Na^+/H^+ antiporter that were able to grow, flower, and produce fruits in the presence of 200 mM Na^+ (Zhang and Blumwald, 2001). Several genes have been identified from *A. thaliana* for enhancing salt tolerance by a complementation approach with yeast mutants using salt overly sensitive (SOS) assays (Hasegawa et al., 2000; Huh et al., 2002). *In vitro* addition of activated SOS2 protein (a protein kinase) increased Na^+/H^+-exchange activity in salt-treated wild-type plants 2-fold relative to transport without added protein (Qui et al., 2002). Although Na^+/H^+-antiporters are responsible for salt tolerance in glycophytes it is unknown whether these proteins are key

elements also in halophytic plants. From the halophyte *Mesembryanthemum crystallinum* the PM MTR1 Na^+/H^+ antiporter was characterized as being involved in salt tolerance (Chauhan et al., 2000), but on the other hand the presence of a strong vacuolar pathway also is known. Na^+/H^+ antiporters have been also identified in some marine archaea and this may indicate a basic mechanism of cytosolic Na^+ regulation (Morsomme et al., 2002; Hellmer et al., 2002). However, probably not a single trait or mechanism make a plant salt tolerant, but rather an interacting network (Patharkar and Cushman, 2000).

One of the best-characterized PM marker enzymes is the H^+-ATPase (Palmgren, 2001). This enzyme is existent in almost all plant cells and allows the identification of this pump by techniques such as ATPase assays. The regulation of ions across the PM is thought to be achieved by an electrochemical gradient generated by PM H^+-ATPase (Palmgren, 2001). Salt treatment increased the H^+-pumping capacity of the PM H^+-ATPase in *Atriplex nummularia* roots (Braun et al., 1986) and PM H^+-ATPase was stimulated in *Salicornia bigelovii* Torr. when the plants were grown in media containing 200 mM NaCl (Ayala et al., 1996). However, it is not clear how H^+-ATPase responds to increasing salinities and whether these changes contribute to the plant's salt tolerance.

We were interested to understand the most important salt tolerance strategies of the halophyte *A. tripolium* which is not only an exciting experimental plant but might also be used as a salt tolerant crop plant. Reported here are the isolation of PM from *A. tripolium* control and 3% NaCl treated plants, a purity check using immunoblotting and subsequent sequencing, and comparisons of enzyme activities of the marker enzyme H^+-ATPase. Also presented are the results of Na^+/K^+ ratio and osmolarity measurements in the NaCl treated plants compared to the control. Since genomic data from *A. tripolium* is not yet available, the protein pattern enabled by two-dimensional gel electrophoresis and subsequent mass spectrometry might help to identify the contributing protein network involved in salt tolerance in halophytes.

6.2 RESULTS

6.2.1 Effect of salt treatment on the phenotype of the *Aster tripolium* plants

A. tripolium plants were grown in soil and then watered with different concentrations of NaCl (0%, 1.5%/257 mM, and 3%/513 mM) for 10 days (refer to chapter 3). The plants were able to grow and finally to flower at NaCl levels between 0 and 3%, close to seawater salinity of 3.5%. The growth rates and therefore the plant sizes were decreased when treated with increasing NaCl concentrations in the watering solution. The leaves of *A. tripolium* plants showed wilting symptoms after 10 days of watering with salt-containing solutions. However, the plants still grew and recovered easily when watered without NaCl in the watering solution. It was demonstrated that NaCl concentrations higher than 3% (tested up to 5%, data not shown) lead to an accelerated senescence and after about 2 weeks to plants' death. The plant species seems to be adjusted perfectly to its ecological niche in the regularly flooded salt marshes. Here short term effects of NaCl on *Aster tripolium* were analyzed; long term experiments investigating already more adapted cells would be a different approach.

6.2.2 Characterization of the plasma membrane (PM) H^+-ATPase marker enzyme from enriched PM vesicles from *Aster tripolium*

Targeted interest was to analyze and understand the mechanisms of salt tolerance in the halophyte *A. tripolium*. The controlled transport of ions across the membranes and the energy status of the cell are major salt tolerance mechanisms (Zhu, 2003). The H^+-ATPases spanning the different membranes in the cell are involved in both ion transport and energy status. Therefore we enriched plasma membranes (PM) and characterized the major marker enzyme of the PM. The isolation procedure of PM differs in dependency of the plant species. For the isolation of PM from the halophyte *A. tripolium* the basic published method was modified as described in methods section. To characterize the isolated PM vesicles from plants watered with 0 and 3% NaCl the protein extracts were analyzed by one dimensional SDS polyacrylamide gel electrophoresis (PAGE), blotted onto nitrocellulose membranes and incubated with an antibody raised against the conserved cytoplasmic region from rice H^+-ATPase (Wada et al., 1992). In parallel the same samples were separated by SDS-PAGE and stained with Coomassie brilliant blue (Fig. 2). The PM proteins of salt treated *A. tripolium* plants could be well separated. The arrow indicates the dominant band of the putative PM H^+-ATPase. The antibody recognized the putative *Aster tripolium* PM H^+-ATPase at about 100 kD in conformity with published results from other plant species (Wada et al., 1992; Muramatsu et al., 2002).

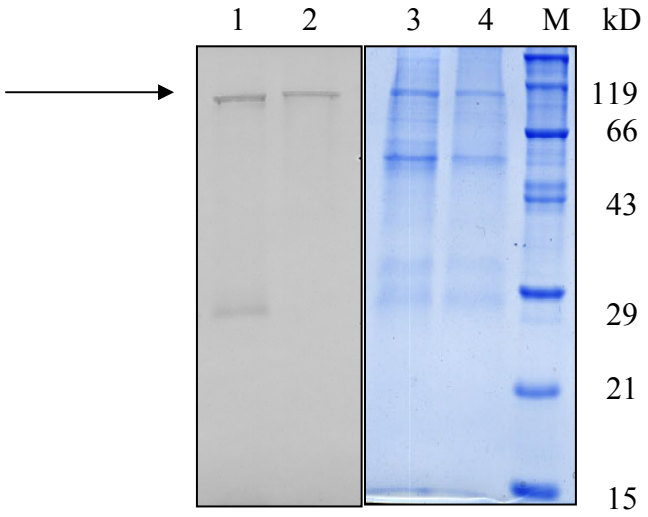


Fig. 2. Immunodetection of P-type H^+-ATPase in the plasma membrane (PM) fraction. Proteins of PM fractions were separated by 12.5% SDS-PAGE, blotted onto a nitrocellulose membrane and incubated with an antibody directed against the conserved cytoplasmic domain of rice PM H^+-ATPase (Wada et al., 1992). Lane 1, 10 µg PM protein fraction from control *A. tripolium* plants; lane 2, 10 µg PM protein fraction from *A. tripolium* plants treated with 3% NaCl in the watering solution for 10 days. On the right hand side the corresponding Coomassie-stained gel is shown (lane 3, 10 µg PM protein fraction from control *A. tripolium* plants; lane 4, 10 µg PM protein fraction from *A. tripolium* plants treated with 3% NaCl in the watering solution for 10 days. The arrow indicates the band of the putative PM H^+-ATPase (about 100 kD).

The steady-state P-type H^+-ATPase levels in treated and untreated *A. tripolium* plants seem to be unchanged; the salt treatment does not induce the expression of higher levels of this ATPase.

6.2.3 Sequencing of plasma membrane H^+-ATPase from *Aster tripolium*

The band at about 100 kD was excised from the Coomassie-stained SDS polyacrylamide gel (Fig. 2) and was sequenced using matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS) and liquid chromatography coupled with mass spectrometry (LC-MS/MS). The peptide sequences obtained were aligned to published peptide databases, and by the high homology to H^+-ATPase sequences from other plant species the protein was identified as *Aster tripolium* H^+-ATPase (Table I). Currently, the encoding DNA sequence is being isolated from *Aster tripolium* using degenerated primers for PCR. The identification and isolation of putative proteins involved in salt defence strategies has to be done in a similar way because there are only very few *Aster tripolium* DNA sequences available in the database.

6.2.4 Capacity of isolated plasma membrane vesicles for H^+-pumping

Next the enzymatic capacity of H^+-pumping and ATP hydrolysis was analyzed to characterize the quality of the PM preparation. Both PM fractions isolated either from control or from salt treated plants were adjusted to the same protein concentration. In each assay 10 µg from the PM protein fraction was used. An 80-90% increase in H^+-pumping and ATP hydrolysis following three freeze-thaw cycles agreed with the findings of Larsson et al. (1987). Proton pump activities of membrane vesicles showed the typical change in acridine-orange fluorescence that follows the application of $MgSO_4$, indicating the formation of a pH-gradient across the vesicle membrane. Incubation of the membrane vesicles with vanadate prior to the application of $MgSO_4$ largely suppressed the formation of this pH-gradient indicating a high abundance of the P-type H^+-ATPase which reacts sensitively to vanadate (Fig. 3A).

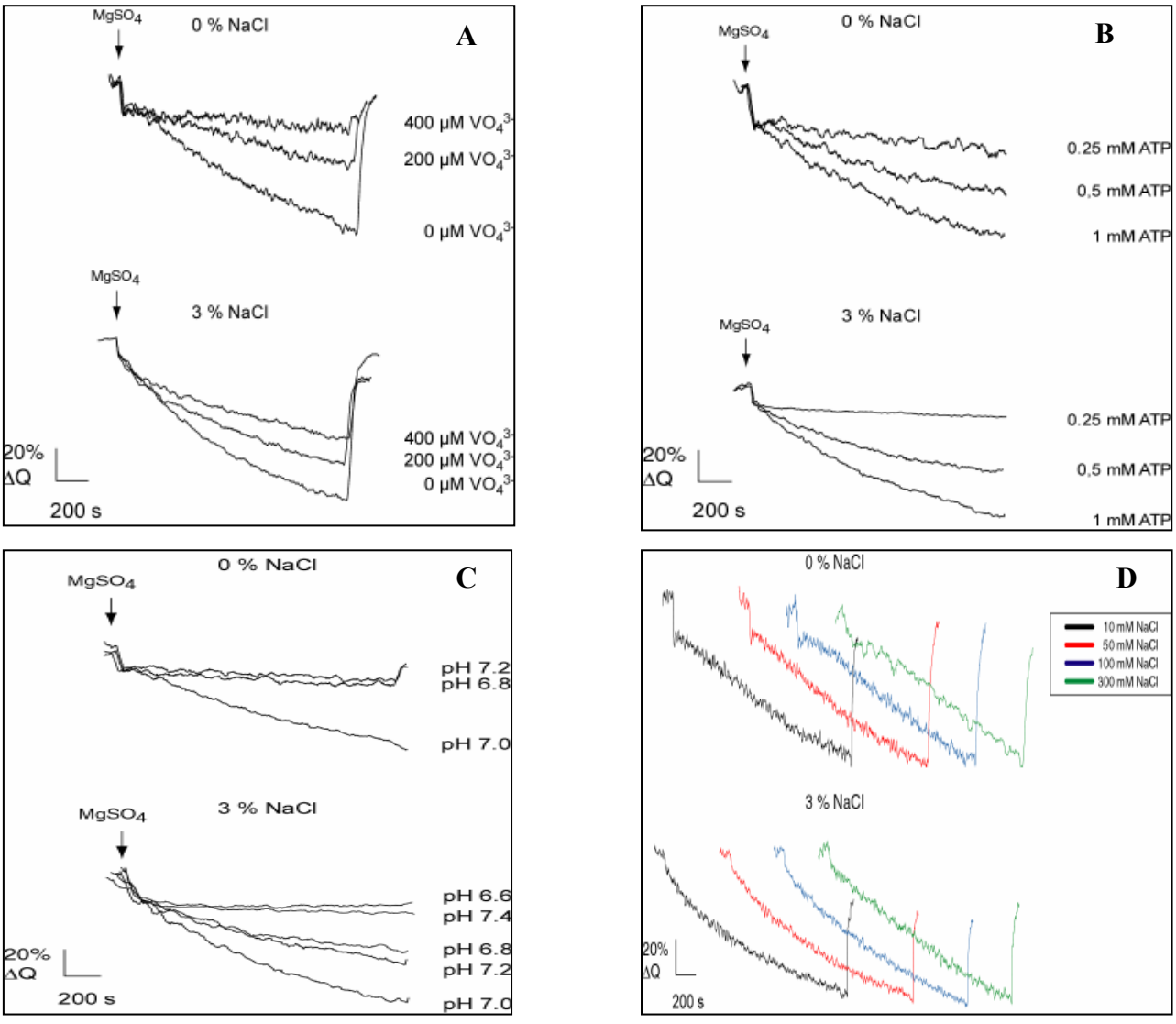


Fig. 3. Characteristics of ΔpH formation determined by the quenching of acridine-orange fluorescence. (A) PM from *Aster tripolium* were enriched according to Larsson et al. (1987) using the phase-partitioning technique with modifications (as described in methods section). The ΔpH was monitored with acridine-orange fluorescence as described by Hellmer et al. (2003). In brief, the formation of the ΔpH was initiated by adding 1.5 mM $MgSO_4$ to the assay mixture and dissipation of the ΔpH was achieved by adding 3 mM NH_4HCl. Traces for vesicles derived from 10 µg inside-out *Aster tripolium* PM-vesicles with 5 mM $MgSO_4$, in presence of Na_3VO_4. (B) Dependence of H^+-pumping on different ATP concentrations. (C) Dependence of H^+-pumping on the pH. (D) Effect of Na^+ on ΔpH of PM-vesicles. Measurements were performed with a UDL200 laser (Laser Technik, Berlin, Germany) using coumarine 102 as laser dye and a MSG801 SD nitrogen laser (Laser Technik, Berlin, Germany) as pump source. Fluorescence of the samples was excited at 493 nm. The emission spectra were dispersed by a MS125 spectrometer (LOT-Oriel-GmbH, Darmstadt, Germany.) and imaged onto an Instaspec IV CCD camera. To monitor H^+-transport the fluorescence intensity at 530 nm was used.

In the presence of increasing concentrations of ATP, uptake and accumulation of H^+ by PM vesicles saturates at 1 mM ATP (Fig. 3B). H^+-pumping in the PM fraction was not affected by nitrate or azide (data not shown). These experiments strongly indicate that H^+-pumping is associated with a P-type H^+-ATPase and enzymes of the V-type or F-type are absent. Uptake and accumulation of H^+ by PM vesicles were optimal when the pH of the extravesicular solution was 7.0 (Fig. 3C). Transport activity was not affected by NaCl concentrations up to 300 mM in the extravesicular solution in both with 0% and 3% NaCl treated *A. tripolium* plants (Fig. 3D). To detect any kinetic differences one might assume for the ATPases in salt stressed *Aster tripolium* plants**,** the K_m values for ATP were calculated. For the determination of the K_m values for the H^+-ATPase in untreated and treated *Aster tripolium* plants 5 ATP concentrations were chosen (0.25, 0.5, 1.0, 3.0, and 7.0 mM ATP). The K_m values were calculated according to the half maximal velocity from Lineweaver-Burk plots. In the PM isolated from the untreated control the K_m for ATP was determined to 1.7 mM and in the PM from *Aster tripolium* watered with 3% NaCl to 4.4 mM. Therefore the efficiency of H^+-pumping is reduced in salt treated halophytic plants.

To investigate the role of PM H^+-ATPase which can be inhibited specifically by vanadate, *Aster tripolium* leaves were incubated in different concentrations of vanadate (1 mM up to 100 mM) without or with 3% NaCl in the medium. The effects of high salt on the leaves were much more pronounced in the presence of vanadate than in 3% NaCl without vanadate (data not shown). The higher concentrations of vanadate damaged the leaves as well. The effects support the role of vanadate-sensitive PM H^+-ATPase in salt tolerance.

6.2.5 Two-dimensional gel electrophoresis of plasma membrane proteins

Since genomic data from *Aster tripolium* are not yet available, our approach was to identify differentially expressed PM proteins in untreated and salt treated *Aster tripolium* plants by two- dimensional (2D) gel electrophoresis and sequence them by mass spectrometry (MS). By comparisons with available peptide databases one should be able to find homologous protein sequences from other species. The proteins from our well characterized PM preparation were first separated by isoelectric focusing (IEF) and for the second dimension they were transferred onto SDS polyacrylamide gels (PAGE). The most critical step was the solubilisation of the PM proteins for subsequent IEF. Several combinations of detergents were applied during optimization of 2D gel electrophoresis (data not shown).

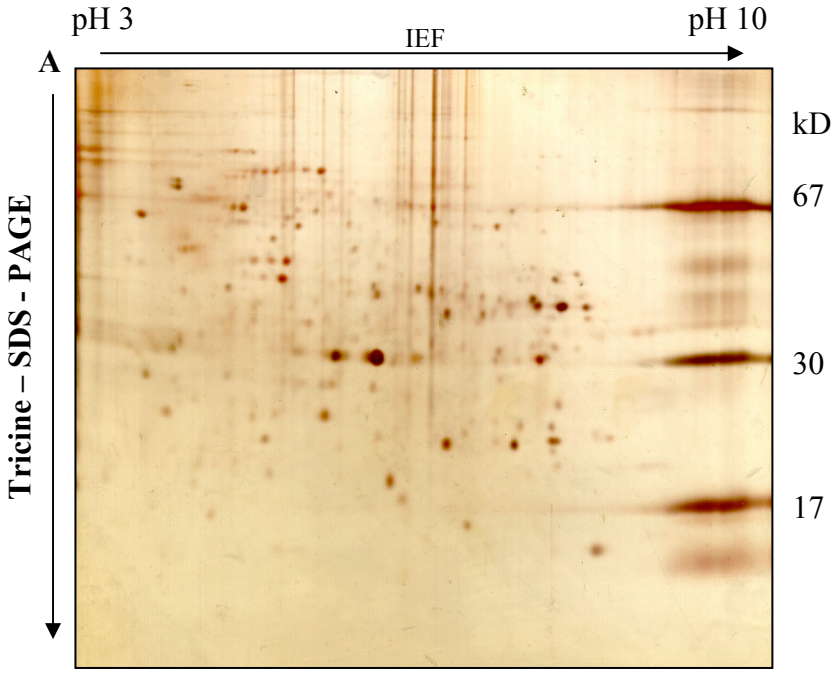


Fig. 4A. Two-dimensional map of the plasma membrane (PM) proteome from *Aster tripolium*. PM proteins were isolated from *Aster tripolium* leaves watered without (A) separated according to their isoelectric points in the range of pH 3 and 10 (first dimension) and their molecular weight (second dimension). The silver-stained gels were loaded with 250 µg protein. Solubilisation of the proteins were carried out using the lysis solution (8 M urea, 4% [w/v] 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 40 mM Tris-HCl, 50 mM DTT) and IEF was carried out using a nonlinear IPG stripe of pH 3 to 10. The marker proteins loaded on the gel were bovine serum albumin (67 kD), myoglobin (30 kD), and cytochrome c (17 kD).

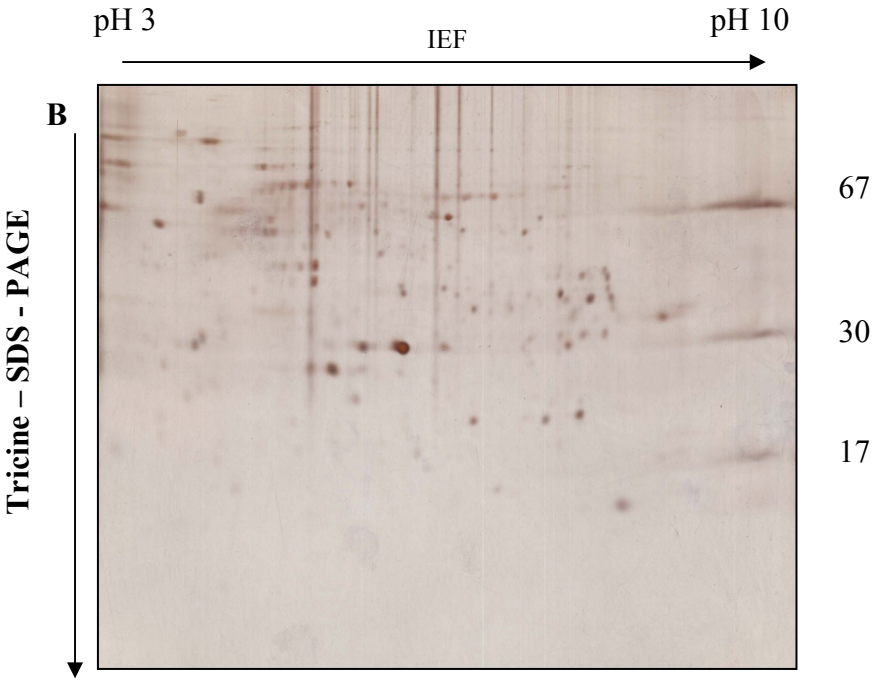


Fig. 4B. Two-dimensional map of the plasma membrane (PM) proteome from *Aster tripolium* (3% NaCl)

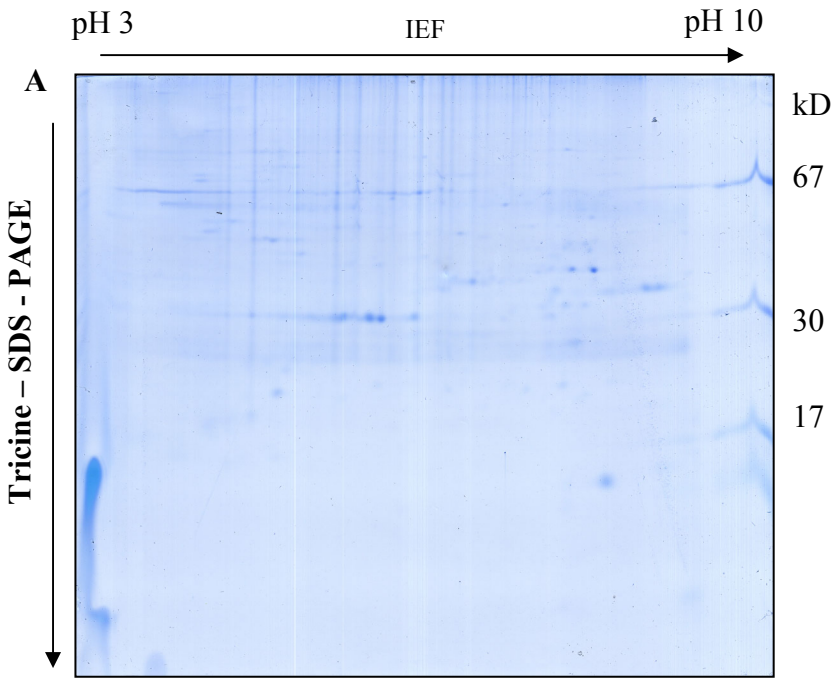


Fig. 5A. Two-dimensional map PM proteome from *Aster tripolium*. PM proteins were isolated from *Aster tripolium* leaves watered without (A) and with 3% NaCl (B). Details are described in the legend to figure 4. The Coomassie-stained gels were loaded with 250 µg protein.

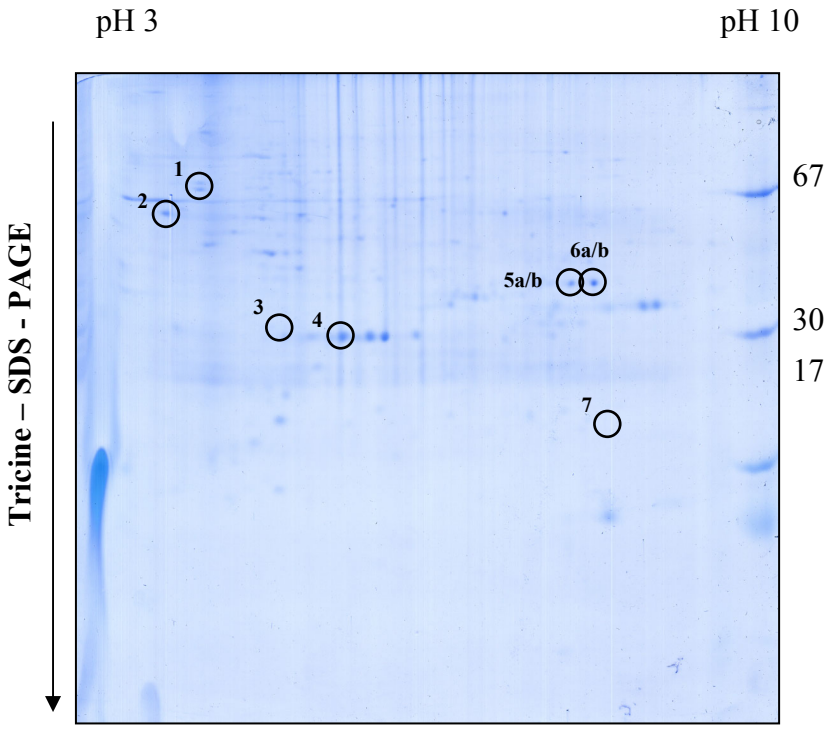


Fig. 5A. Two-dimensional map PM proteome from *Aster tripolium*. (3% NaCl) The protein spots surrounded by a circle show different abundances in the PM proteome of untreated and salt-treated *A. tripolium* leaves. Selected protein spots were analyzed by mass spectrometry. Spot numbers correspond to the numbers in Table I.

The best results with respect to yield and reproducibility were obtained with the procedure described in the material and methods part. In figure 4 and figure 5 representative gels of the PM proteins from untreated *Aster tripolium* (Fig. 4A and 5A) and salt treated plants (Fig. 4B and 5B) are shown. To detect as many differentially expressed proteins as possible the gels were silver-stained (Fig. 4). In figure 5 the PM proteins isolated from the same plant material were separated and the gels were Coomassie-stained. Only Coomassie-stained protein spots could be digested by trypsin and analyzed by MS.

Table I. Identified proteins of the plasma membrane proteome from *Aster tripolium*

No[a]	Accession[b]	Protein name	pI[c]	Mass[c]	Identification[d]	Sequence[e]
1	Q37160	Ribulose-1,5-bisphosphate carboxylase, large subunit	6.09	53.8	LC-MS/MS	LTYYTPEYETK
2	Q40040	Calreticulin	4.45	47.0	LC-MS/MS	FYAISAEYPEFSNK
3	Q9BAT4	Ribulose-1,5-bisphosphate carboxylase, large subunit (fragment)	6.53	46.7	MALDI-TOF	LTYYTPEYETK
4	Q8RWW2	Putative carbonic anhydrase	5.54	29.5	MALDI-TOF	FMVFACSDSR
5a	P45623	Delta-aminolevulinic acid dehydratase	5.72	45.1	LC-MS/MS	LAAALIER
5b	P46248	Aspartate aminotransferase	8.18	49.8	LC-MS/MS	VSVEPSPR
6a	Q9sdt1	Protochloro-phyllide reductase	9.20	43.3	LC-MS/MS	WHVIMACR (MetOx)
6b	Q42435	Capsanthin/ capsorubin synthase	8.77	56.6	LC-MS/MS	ETLLKPFPLLSIPTPN MYSFKHNSTFPNPT-K
7	Q9ayq8	Ubiquitin (fragment)	8.58	11.3	LC-MS/MS	IQDKEGIPPDQQR
*	Q9m2a0	Plasma membrane	5.53/	104/	MALDI-TOF	TGTLTLNK

Q9m461	H^+-ATPase	6.80	105	and LC-MS/MS

[a]The numbers refer to the spots as given in Figure 4D. [b]The accession numbers represent protein entries. [c]Calculated molecular masses and isoelectric points are given for proteins with known mature N-termini. [d]Methods of protein identification: MALDI-TOF, Matrix-Assisted Laser Desorption Time-Of-Flight Mass Spectrometry and LC-MS/MS, Liquid chromatography coupled with mass spectrometry. [e]Partial amino acid sequences as determined by electrospray ionization tandem mass spectrometry. [*]Refers to the band excised from the 1D Coomassie gel of Fig. 2.

The reproducibility of the PM protein patterns has been established by repeating the 2D gel electrophoresis 8 times. Each staining method, with silver and with Coomassie, was applied 4 times resulting on average of about 175-200 protein spots on the silver stained gels and 80-90 on the Coomassie-stained gels. When comparing the patterns between PM proteins from untreated and salt treated *Aster tripolium* plants a number of up- and down-regulated proteins could be detected (estimated about 20%). 10 proteins spots were cut for sequence analysis. In 7 cases the MALDI-TOF MS analysis gave no signal; therefore LC-MS/MS was used to obtain peptide sequence information (Table I). Several soluble protein were up-regulated by the salt treatment, such as ribulose-1,5-biphosphate carboxylase oxygenase (RubisCO), carbonic anhydrase, calreticulin, protochlorophyllide reductase, capsanthin/capsorubin synthase, and ubiquitin (Table I).

6.2.6 Na^+ and K^+ contents in *Aster tripolium* leaves

Information about Na^+ to K^+ contents in *Aster tripolium* in this study would be useful in estimating uptake selectivity in which the PM H^+-ATPase role must be involved. Na^+ and K^+ contents in *Aster tripolium* leaves were determined at day 5 by atomic absorption spectrometry (AAS). In the controls watered with tap water only (electrical conductivity about 0.8 dS m^{-1}, Na^+ content about 40 mg L^{-1}) the Na^+ accumulated to about 1 mmol per g dry weight (Table II). The higher the NaCl concentrations in the watering solution the higher the Na^+ contents in the leaves. However, the Na^+ in the leaves did not increase direct proportional to the NaCl in the watering solution. The ratio of Na^+ to K^+ was 0.65 in the leaves of the salt treated plants after 5 days of treatment in comparison to a quotient of 0.33 in the controls (Table II). In addition, the osmolarity of the leaves was measured. One day after beginning of the treatment the osmolarity was about 10% increased in the leaves of the salt treated plants in comparison to controls whereas after 5 days the osmolarity was almost as double as high as in

the control plants (Table II). The water content of about 90% in the *Aster tripolium* leaves remained almost constant under all conditions investigated. Taken the results together one has to assume an active exclusion mechanism for both Na^+ and K^+. The PM H^+-ATPase might play an important role in the exclusion of Na^+ and K^+ in *Aster tripolium*.

Table II. Determination of ion contents and of the osmolarity in *Aster tripolium* plants
The contents of Na^+ and K^+ were measured in the leaves of *Aster tripolium* plants grown in 0%, 1.5%/257 mM, and 3%/513 mM NaCl concentrations in the watering solution after 5 days of the treatment by atomic absorption spectrometry. The osmolarity was determined in the same plant material using a freezing point osmometer at day 1 and day 5.

NaCl conc.	Concentration of Na^+ (mmol g DW^{-1})	Quotient of ions Na^+/K^+	Osmolarity (mOsmol kg^{-1})	
	day 5		day 1	day 5
0%	0.97±0.06	0.33	386±94	389±98
1.5%	1.58±0.20	0.52	431±95	513±11
3%	2.40±0.12	0.65	443±98	615±53

6.3 DISCUSSION

In plants and thermophilic archaea PM H^+-ATPase acts as the primary transporter that pumps protons out of the cell, thus creating a pH and an electrical potential gradient across the PM that in turn activates many secondary transporters involved in ion and metabolite uptake (Serrano, 1989; Sussman, 1994; Sze et al., 1999; Palmgren, 2001). From several glycophytic plants it is known that elevated cytosolic Na^+ inhibits H^+-pumping activity and its importance in salt tolerance has been suggested through various studies in glycophytes (Douglas and Walker, 1984; Ben-Hayyim and Ran, 1990). Several strategies might be used by *A. tripolium* to tolerate high NaCl concentrations in the medium by hindering Na^+ to enter the cell. The main interest was to analyze the role of PM proteins in salt tolerance mechanisms of this halophyte.

To characterize the kinetic properties and to examine whether Na^+ inhibits H^+-pumping activity PM enriched fractions were isolated from *Aster tripolium* leaves. Preparation of PM vesicles from plants differs with each plant species. After optimization the PM isolation

yielded in good quality right-side-out PM vesicles. The presence and abundance of the 100 kD PM marker enzyme P-type H^+-ATPase has been demonstrated by immunoblotting (Fig. 2). Three times freeze-thawed PM vesicles were used to study the enzyme activity of PM H^+-ATPase. Purity of the PM vesicles was proven by inhibition assays with vanadate in the extravesicular medium (Fig. 3A).

The optimal substrate concentration was 1 mM ATP (Fig. 3B), the optimal pH of the ATP-dependent acidification of the vesicle lumen catalysed by *Aster tripolium* PM H^+-ATPase activity was pH 7.0 (Fig. 3C). The specific activity of PM H^+-ATPase was the same in PM isolated from untreated and salt treated plants. The NaCl inhibition experiments with the PM H^+-ATPase from 0% and 3% treated *Aster tripolium* showed no changes to NaCl addition in extravesicular medium of measurement up to 300 mM (Fig. 3D). The K_m values for ATP of PM H^+-ATPase from *Aster tripolium* of 1.7 mM (0% NaCl) and 4.4 mM (3% NaCl) indicate that the enzyme is less specific for its substrate at high in salt stress.

In comparable studies two monocotyledonous plant salt tolerant species were investigated and in compared to monocotyledonous glycophytes. In the case of the C_4 seagrass *Spartina patens* (Aiton) Muhl callus was used for the preparation of PM (Wu and Seliskar, 1998), the marine seagrass *Zostera marina* L. plants were collected from the coast (Muramatsu et al., 2002). In *S. patens* there was a significant increase up to 2,5-fold of PM H^+-ATPase activity during salt treatment but the amount of H^+-ATPase on the PM did not change as demonstrated by immunoblotting (Wu and Seliskar, 1998). In the cells a drop of the K_m values for ATP from 3.6 mM to 2.4 mM was caused by a salt treatment 340 mM in the medium compared to controls (Wu and Seliskar, 1998). Interestingly, this enzyme activity was inhibited non-competitively by NaCl *in vitro*. The response of PM H^+-ATPase activity in *S. patens* suggests that this species has evolved mechanisms that can adapt the activity of this enzyme when cells are exposed to NaCl. A salt-tolerant PM H^+-ATPase was identified in *Z. marina* whereas other enzymes in the organism were sensitive to salt. The Na^+ concentration was very low inside *Z. marina* cells leading to the assumption that the PM salt-tolerant ATPase is involved in maintaining low Na^+ concentrations (Muramatsu et al., 2002), a typical strategy for excluders. In the same study it was shown that the PM H^+-ATPases from glycophytes were inhibited by NaCl, already at low NaCl concentrations (Muramatsu et al., 2002). One cannot generalize the properties of PM H^+-ATPase in halophytes or at least in halophytic monocots and dicots but has to investigate each species separately.

The family of the P-type ATPases in plants can be divided into 5 subfamilies. In *Arabidopsis thaliana* 46 members have been identified, 11 belong to the subfamily of

autoinhibited H^+-ATPases which can be regulated by phosphorylation at the C-terminus (Baxter et al., 2003). An *Arabidopsis thaliana* T-DNA insertion mutant of one member, *AtAHA4*, confers salt sensitivity. The respective gene is expressed in the root endodermis, in flowers, and in maturing siliques (Vitart et al., 2001). However, the data collected so far indicate strong species specific differences between subcellular localization and tissue specificity, and therefore function in the organism which makes the transfer to other species difficult. There are already some sequences of PM H^+-ATPases from halophytic (*Mesembryanthemum crystallinum, Zostera marina*) species in the databases available. The protein sequences of around 950 amino acids from different species show high levels of identity and similarity. We obtained the highest scores of similarity for a glycophytic (*Nicotiana tabacum*) and a halophytic species (*Mesembryanthemum crystallinum*). Therefore one could ask the question what makes the difference in this particular protein between halophytes and glyocophytes? One could speculate about different single amino acids residues which influence stability and activity in the three-dimensional structure or about conformational changes during salt stress. The recent advances in modeling of the 3D-structures of P-type H^+-ATPases (Kühlbrandt et al., 2002) might help to understand the special properties of these enzymes in halophytes.

The existence of a H^+-ATPase in the PM of *Zostera marina* indicates protons as the major driving ions for transport. However, the existence of a H^+/Na^+ antiporter and, therefore, the maintenance of a high inwardly directed electrochemical gradient for Na^+, also suggest that Na^+ could play this role in *Zostera marina* (Fernandez et al., 1999). This can be further explained by the theory that V-type H^+-ATPases exclusively catalyze the ATP-driven export of protons, the different P-type H^+-ATPases can transport other cations as well. It has been found that ATPases vary their apparent K_m for ATP with the size of the membrane potential and the ion transport rate. Here, it would be reasonable to think about PM H^+-ATPase of 3% *Aster tripolium* as PM Na^+-ATPase which would be an interesting investigation in future studies. It would be also interesting to investigate the abundance and activity of Na^+/H^+ antiporters in *Aster tripolium* by antibody and enzyme assay studies.

Currently, we are trying to amplify PM H^+-ATPase from *Aster tripolium* using degenerated primers according to the sequences given in Table I and from alignment data of PM H^+-ATPase sequences from different species. *In vitro* reconstitution of enzyme activity using recombinant PM H^+-ATPase would be an interesting approach to characterize the kinetic data in more detail. In preliminary microarray results using the heterologous system of *A. thaliana* EST clones and *Aster tripolium* probes the expression of a homologue to the V-

type H^+-ATPase from *Arabidopsis thaliana* was highly induced at high salt (data not shown). Therefore the identification and isolation of specific vacuolar H^+-ATPases might be good target proteins for improving salt tolerance in halophytes and glycophytes.

The separation of PM proteins from *Aster tripolium* leaves by 2D gel electrophoresis could be established through this study. The resulting protein patterns were comparable between different experiments and between the different salt treatments (Fig. 4 and Fig. 5). However, the amount of separated protein spots is low in comparison to the predicted size of the PM proteome of higher plants, about 500 polypeptides for the model plant *Arabidopsis thaliana* and approximately 750 polypeptides in the case of species with larger and more complex genomes (Masson and Rossignol, 1995). In the silver-stained gels of PM polypeptides from *Aster tripolium* about 175-200 proteins could be visualized. Obviously many PM proteins were lost during the process of IEF and SDS-PAGE or already during the PM preparation. The solubilisation of the hydrophobic proteins out of the PM might not have been complete also indicated by the absence of the very abundant H^+-ATPase visible in 1D gel electrophoresis. Some proteins (estimated 11-16%) might have been lost during IEF procedure using immobilized pH gradients (IPGs) due to the absorption to the matrix (Rabilloud et al., 1997). Many proteins with extreme properties (hydrophobic, acid, basic, small, large) often remain in the IPG stripes and can not be transferred to the SDS gel. In halophytes more proteins with these extreme characteristics might occur. The major problem was to obtain enough PM of high quality for optimization of the 2D gel electrophoresis conditions and for the final protein sequencing experiment. It was calculated that one would need about 5-10 mg of PM protein to separate more than 75% of the proteins with more than 50 pmol per spot for sequencing. It was impossible to solubilize these high amounts of protein for loading of one gel.

In experiments done previously with *Aster tripolium* salt-adapted cell lines show the profiles of total extracts divided in soluble and insoluble proteins separated by 2D and 1D gel electrophoresis, respectively. Several proteins were up- and others were down-regulated by a salt treatment, however, their identity was not determined (Uno et al., 1996). In total extracts it will be almost impossible to detect all proteins. Also the physiology of cell cultures differs from green plants, and subproteoms isolated from cell cultures are not comparable to the respective subproteoms from green plants which make this experimental system difficult to compare.

In our study, about one third of proteins detected by silver-staining were up- or down regulated by the salt treatment. The protein identification obtained by MS sequencing indicate

according to the classification by Santoni et al. (1999), that a preparation enriched in PM proteins was successful. Because of limited MS access only 10 proteins up-regulated by salt could be sent for sequencing (Table I). Some of them could not be identified because of the small amount or because of hydrophobicity.

It was reported by Santoni et al. (1998) and Kawamura and Uemura (2003) that a majority of the *Arabidopsis thaliana* PM proteins separated on 2D gel electrophoresis did not possess transmembrane domains suggesting solubilized fractions separated on 2D gel electrophoresis are peripherally and/or non-covalently bound to the PM. Although the PM fractions were proven to be pure through molecular marker enzymes, there might be still the possibility that some proteins come from intracellular membranes that are included in the PM fraction (Kawamura and Uemura, 2003). On the other hand, Berczi and Asard (2003) underline that the soluble and non-specifically bound contaminants can be significant in PM enriched fractions. Therefore conclusions about the localization of a PM protein can only be drawn when the correct homogenization and/or stripping conditions have been used.

The proteins that increased during NaCl stress in Aster tripolium included those required for CO_2 fixation. RubisCO and carbonic anhydrase were those proteins identified in this study as significantly up-regulated. Carbonic anhydrase plays a crucial role in CO_2-concentrating mechanism (Badger and Price, 1994) and is known to be located in cytoplasm as well as in chloroplast (Rumeau et al., 1996). Calreticulin, a protein known for its function as calcium-binding molecular chaperone, and hypothesized to be involved in ion homeostasis during environmental disturbance (Nelson et al., 1997) (in this case salt stress) also has been identified.

Four mechanisms to prevent Na^+ accumulation in the cytoplasm would be: reducing Na^+ entry into the cell, active Na^+ efflux from the cell, active sequestration of Na^+ in the vacuole and osmotic adjustment in the cytoplasm. Through this study on *Aster tripolium*, we have demonstrated that PM H^+-ATPase is having specific functions in halophytes which could not be found in glycophytes, further research in expressing *Aster tripolium* PM H^+-ATPase would reveal the role played in the cell barrier to help the plant devoid of toxic Na^+ ions.

The analysis of salt overly sensitive (SOS) *Arabidopsis thaliana* mutants gave the basis for a model to explain salt tolerance in the glycophyte *Arabidopsis thaliana* (Qui et al., 2002). SOS3, a calcium sensor, activates SOS2, a protein kinase, that in turn phosphorylates and activates SOS1, a PM Na^+/H^+ antiporter. *SOS1* mRNA is stabilized and accumulates under salt stress conditions, suggesting post-transcriptional control of *SOS1* transcript accumulation. In response to NaCl treatment, transgenic plants overexpressing *SOS1* accumulated less Na^+

in the xylem transpirational stream and in the shoot. Conferring salt tolerance through limiting Na^+ accumulation in plants through Na^+ efflux and export of Na^+ to soil by root epidermal cells (Shi et al., 2003).

A recently published report on an investigation between PM H^+-ATPase (PMHA) and PM Na^+-ATPase (PMNA) showed large variation between K_m values experimented in marine alga *Tetraselmis viridis* (Pagis et al., 2003). Taking this view it can be questioned whether the reported ATPase from *Aster tripolium* of plants treated with 3% NaCl might be PM Na^+-ATPase. In that case more research is needed in *Aster tripolium* grown with NaCl in the watering medium. It was demonstrated by Apse et al. (1999) that the expression of a vacuolar Na^+/H^+ antiporter in *Arabidopsis thaliana* lead to sustained growth and development of the transgenic plants at salt concentrations in the soil up to 200 mM NaCl. A vacuolar salt accumulation was observed.

The main strategy for the salt marsh plant *Suaeda salsa* L. was reported to be the up-regulation of V-type ATPase activity in tonoplast vesicles but by an increase in V-type ATPase protein amount (Wang et al., 2001). A model proposing how ion homeostasis is achieved in *Mesembryanthemum crystallinum* under salt stress is outlined by Sul et al. (2003). In leaves up-regulation of SOS1, HKT, a calcium transporter, and HAK, a potassium transporter in the PM, and of the NHX antiporter in the tonoplast facilitate uptake and accumulation of Na^+ into the vacuole of leaf cells. A leaf-specific Na^+/inositol symporter expressed in the tonoplast is down-regulated thus reducing Na^+ leakage from vacuoles (Adams et al., 1992; Chauhan et al., 2000; Barkla et al., 2002; Yokoi et al., 2002). Therefore a network of transporters probably mediates salt tolerance regulated by protein kinases, in the case of PM H^+-ATPase by 14-3-3 proteins.

An other adaptation to salt might be an increase in the volume of the vacuole due to accumulation of ions and osmolytes (Mimura et al., 2003). Currently, investigations are underway to investigate the concentrations of ions in different compartments of *Aster tripolium* by SEM-EDX analysis because it would be very helpful to know also the distribution of ions between the vacuole and the cytoplasm and between the apoplast and the symplast. The synthesis and accumulation of osmolytes to prevent damage by Na^+ is also realized in *Aster tripolium* as demonstrated by the over-proportional increase in osmolarity during salt treatment.

Probably all four strategies to exclude Na^+ from the cytoplasm are realized in halophytes to make them salt tolerant to a certain degree. The expression of all gene products involved in salt tolerance in glycophytes will be a difficult task. Therefore the use of already salt tolerant

halophytes as crop plants might be a more successful way. To understand the basic mechanisms of salt tolerance in halophytes on the molecular level might help to select the most suitable ecotypes.

7 Transcriptome variation in *Aster tripolium* due to salt stress

7.1 INTRODUCTION

Abiotic stresses can severely impair plant growth and performance. Environmental factors, such as drought, extreme temperatures or high and fluctuating salinity, are responsible for significant yield reductions in cultivated areas world-wide (Ozturk et al., 2002). Understanding abiotic stress tolerance, not to mention breeding for stress tolerance, proved difficult because of the trait's multigenicity. As a consequence no traditional crop lines exist that combine tolerance to high salinity or drought with high yield, which represents yet another complex trait (Bohnert et al., 2001). A large and increasing number of genes, transcripts and proteins have been correlatively implicated in stress response pathways, while their precise functions in either tolerance or sensitivity often remain unclear (Bray, 1997). Recently, microarray technology has become a useful tool for the analysis of genome-scale gene expression (Eisen and Brown, 1999; Schena et al., 1995). However, despite widespread acceptance, the use of microarrays as a tool to better understand processes of interest to the plant physiologist is still being explored (Wullschleger and Difazio, 2003). Implemented in the context of a well-designed experiment, microarrays provide high-throughput, simultaneous analysis of mRNA for hundreds, if not thousands, of genes (Aharoni, 2002). *Arabidopsis thaliana* has been used as a model plant system for studying all aspects of plant development and physiological responses (Meyerowitz et al., 1991), and considerable effort has been directed at developing molecular tools for this species (The Arabidopsis Genome Initiative, 2000). It is not known how, or if, these *Arabidopsis*-specific tools may be used to study less characterized plant systems. However, conserved ortholog marker sets for dicotyledonous plants have been identified (Fulton et al., 2002), suggesting that hybridisation-based tools, such as the microarrays, offer greatest possibility for the study of heterologous systems (Horvath et al., 2003). Though a number of well-characterized experiments on differentially expressed genes during salt-stress were reported (Kreps et al., 2002; Ozturk et al., 2002; Seki et al., 2002; Kawasaki et al., 2001), all have been experimented in glycophytes with the exception of *Mesembryanthemum crystallinum*, a halophytic land plant (Bohnert et al., 2001). Therefore it was a challenge to investigate the coastal plant *Aster tripolium* using this techniques which could also serve for the investgation of parallel expression of homologous genes. This study was performed with RNA extracted from the salt-marsh plant *Aster tripolium* hybridised to the EST clones of *Arabidopsis thaliana*.

7.2 RESULTS

7.2.1 Hybridization of heterologous RNA from 0% and 3%/513 mM NaCl treated *Aster tripolium*

Aster plants were grown in soil and then watered with different concentrations of NaCl (0%, 1.5%/257 mM, and 3%/513 mM) for up to 10 days (refer to chapter 3 for a detailed physiological description). The plant material used for the experiment was from day 3, because the idea was to understand the initial salt stress effect in *Aster*.

Arabidopsis cDNA microarrays were used to study the transcriptomes from leaves of these *Aster* plants. Atleast two biological replicates of samples for 0% and 3%/513 mM NaCl treated *Aster* were hybridised to arrays. Over 41% of the 16,128 cDNA clones present on the Michigan State University (East Lansing) collection micoarrays consistently produced a signal greater than the conditions for the threshold level. These values represent a minimum percentage of hybridising clones as other hybridised might not been recognized as having right threshold conditions or in other words were not represented in all the hybridisation experiments.

7.2.3 Differential expression to heterologous cDNAs from *Aster tripolium*

Around 2.6% of the hybridising genes were statistically differentially expressed. Among these genes around 1% were upregulated at least two fold (Table II) and 1.5% of the expressed genes were downregulated (Table III). An overview of the filter design was represented in figure 1 and in table I. Analyses of the hybridisations indicate that 174 genes were identified as being differentially expressed when the transcriptomes of leaves compared from *Aster* 0% to 3%/513 mM NaCl watered plants.

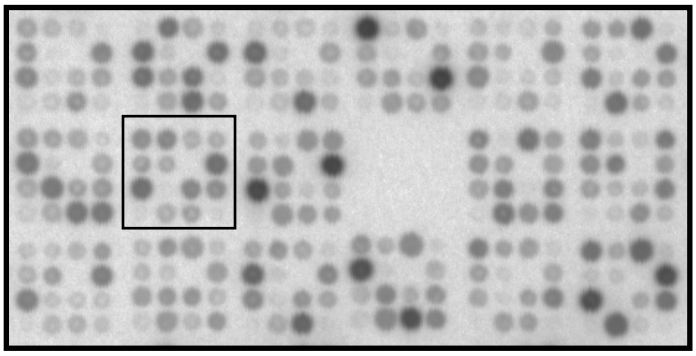


Fig. 1 Design of a microarry filter having *Arabidopsis* EST clones and hybridised with *Aster* RNA. Each filter has 48x48=2304 large squares, each square has 4x4=16 spots totalling 2304x16=36864 spots. Each clone was spotted twice for reproducibility finally having 36864:2=18432 clone pairs. Inner square denotes one big square.

Table I Overview of the differential expression of genes in *Aster*.

Total number of genes hybridized	6643
Statistically differentially expressed genes	174 (~2.6%)
Upreulated genes	69 (~1%)
Downregulated genes	100 (~1.5%)

Careful analysis indicated that most of the genes differentially expressed were recognizably involved in photosynthesis namely carbonic anhydrase, chlorophyll a-b binding protein, putative thioredoxin, putative RubisCO subunit-binding protein and phosphate/phosphonenolpyruvate precursor. Also a number of genes differentially expressed were involved not only in salt stress but in variety of abiotic stress regulation namely putative aquaporin, osmotin precursor, H^+-transporting ATPase, polyubiquitin and putative abscisic acid responsive elements. Cysteine synthase gene involved in sulfur metabolic network is down regulated.

7.2.4 Northern analysis of putatively differentially expressed genes

Homologs of 4 genes (At4g38510, At5g66400, At4g34870 [Table II], At4g11650 [Table III]) which were identified as differentially expressed by heterologous microarray anaylsis were used for Northern blotting. Probable H^+-transporting ATPase (At4g38510) was selected based on its importance in salt stress and osmotin (At4g11650) was selected based on its function of osmolarity during salt stress. The other two dehydrin RAB18-like protein (At5g66400) and peptidylprolyl isomerase (cyclophilin) (At4g34870) were selected due to its annotation related to generalized stress functions. But the first heterologous hybridisation was unsuccessful with all the four *Aster* RNA blots failing to hybridise with *Arabidopis* probes. But the process is ongoing to establish the right conditions for functioning of the Northern blot.

Table II Genes that hybridized preferentially to labelled cDNA and were upregulated in 3%/513 mM *Aster tripolium* in comparison to the 0% plants.

Accession No.	Annotation	P < 0.05	Relative fold induction
AT5G44080	putative protein	0.05	4.91
AT1G59740	hypothetical protein	0.05	3.72
AT5G14740	CARBONIC ANHYDRASE 2	0.03	3.70
*AT4G38510	probable H+-transporting ATPase	0.04	3.32
AT5G65590	DOF zinc finger protein-like	0.04	3.09
AT1G49580	CDPK-related protein kinase, putative	0.04	3.08
AT1G62790	unknown protein	0.05	3.03
*AT5G66400	dehydrin RAB18-like protein (sp|P30185)	0.02	3.01
AT1G68100	unknown protein	0.04	2.96
AT1G75950	skp1-related protein	0.04	2.95
AT3G23170	unknown protein	0.02	2.84
AT1G01730	unknown protein	0.04	2.82
AT4G05320	polyubiquitin (ubq10)	0.03	2.82
AT1G12920	eukaryotic peptide chain release factor subunit 1,	0.03	2.73
AT2G04170	unknown protein	0.02	2.71
AT1G07960	unknown protein	0.03	2.68
AT3G47470	CHLOROPHYLL A-B BINDING PROTEIN 4 PRECURSOR homolo	0.01	2.62
AT3G42670	putative protein	0.05	2.62
AT4G17640	casein kinase II beta chain CKB2	0.04	2.59
AT1G53210	hypothetical protein; similar to ESTs gb|N37409.1,	0.03	2.54
AT5G17870	plastid-specific ribosomal protein 6 precursor (Ps	0.03	2.54
AT4G05070	coded for by A. thaliana cDNA T44741	0.04	2.53
AT2G37940	unknown protein	0.04	2.52
AT3G19290	putative abscisic acid responsive elements-binding	0.05	2.51
AT3G21720	putative isocitrate lyase	0.05	2.51
AT5G38420	ribulose bisphosphate carboxylase small chain 2b	0.03	2.49
AT5G27000	kinesin-like heavy chain	0.05	2.48
AT1G65430	24014845	0.03	2.45
AT2G29020	unknown protein	0.03	2.43
*AT4G34870	peptidylprolyl isomerase (cyclophilin)	0.03	2.43
AT2G17130	putative NAD+ dependent isocitrate dehydrogenase s	0.05	2.43
AT1G67470	putative protein kinase	0.05	2.43
AT1G36310	hypothetical protein	0.05	2.41
AT4G19120	putative protein	0.04	2.40
AT1G08570	putative thioredoxin	0.04	2.38
AT1G57860	hypothetical protein	0.05	2.32
AT1G27390	putative protein import receptor	0.00	2.32
AT4G21750	L1 specific homeobox gene ATML1/ovule-specific hom	0.04	2.31
AT5G02960	putative protein	0.05	2.30
AT5G17920	5-methyltetrahydropteroyltriglutamate--homocystein	0.04	2.29
AT2G35680	unknown protein	0.04	2.28
AT1G30120	hypothetical protein	0.00	2.26
AT1G53580	glyoxalase II	0.05	2.24
AT5G36880	acetyl-CoA synthetase	0.04	2.24
AT5G25210	putative protein	0.03	2.22
AT5G18480	putative protein	0.04	2.22
AT5G59780	MYB27 protein - like	0.01	2.22
AT4G12230	putative protein	0.04	2.22
AT4G15000	ribosomal protein	0.03	2.22
AT5G59790	putative protein	0.03	2.20
AT5G46900	extA (emb|CAA47807.1)	0.01	2.18
AT5G15230	GASA4	0.02	2.18
AT3G07630	putative P-protein: chorismate mutase, prephenate	0.02	2.18

AT2G25070	putative protein phosphatase 2C	0.05	2.17
AT5G26610	putative protein	0.02	2.13
AT5G11800	putative potassium transport protein	0.04	2.13
AT1G11260	glucose transporter	0.04	2.12
AT5G23340	putative protein	0.03	2.09
AT3G53420	plasma membrane intrinsic protein 2a	0.05	2.08
AT3G08580	adenylate translocator	0.03	2.07
AT1G23290	60s ribosomal protein l27a.	0.00	2.06
AT4G18730	ribosomal protein L11, cytosolic	0.05	2.06
AT3G07390	unknown protein	0.04	2.04
AT3G48000	aldehyde dehydrogenase (NAD+)-like protein	0.05	2.04
AT1G56220	hypothetical protein	0.04	2.04
AT5G44550	putative protein	0.03	2.03
AT4G09730	putative protein	0.05	2.02
AT4G18360	glycolate oxidase - like protein	0.05	2.02
AT3G05670	unknown protein	0.03	2.00

***** Genes selected for Northern blotting

Table III. Genes that hybridized preferentially to labelled cDNA and were downregulated in 3%/513 mM *Aster tripolium* in comparison to the 0% plants.

Accession No.	Annotation	P < 0.05	Relative fold induction
AT1G13080	putative cytochrome P450 monooxygenase	0.03	0.50
AT5G02500	dnaK-type molecular chaperone hsc70.1	0.03	0.50
AT2G16850	putative aquaporin (plasma membrane intrinsic prot	0.01	0.50
AT2G35790	unknown protein	0.02	0.50
AT5G19510	elongation factor 1B alpha-subunit	0.02	0.50
AT2G28000	putative rubisco subunit-binding-protein alpha sub	0.03	0.50
AT3G13920	eukaryotic protein synthesis initiation factor 4A	0.04	0.49
AT5G58070	outer membrane lipoprotein - like	0.02	0.49
AT2G29590	unknown protein	0.03	0.49
AT3G53020	60S ribosomal protein - like	0.03	0.49
AT4G03030	predicted OR23 protein of unknown function	0.01	0.48
AT1G09570	putative phytochrome A	0.03	0.48
AT1G55360	unknown protein	0.01	0.48
AT1G66970	hypothetical protein	0.00	0.47
AT3G61440	cysteine synthase AtcysC1	0.03	0.47
AT1G80920	J8-like protein	0.05	0.47
AT2G40140	putative CCCH-type zinc finger protein	0.05	0.47
AT3G61870	putative protein	0.01	0.47
AT3G47340	glutamine-dependent asparagine synthetase	0.01	0.46
AT1G31850	hypothetical protein	0.04	0.46
AT5G49980	transport inhibitor response 1 protein	0.02	0.46
*AT4G11650	osmotin precursor	0.04	0.46
AT3G51370	protein phosphatase 2C -like protein	0.02	0.46
AT2G26300	G protein alpha subunit 1 (GPA1)	0.04	0.46
AT1G67090	hypothetical protein	0.03	0.46
AT4G38920	H+-transporting ATPase 16K chain P2, vacuolar	0.05	0.46
AT3G02230	reversibly glycosylated polypeptide-1	0.05	0.45
AT5G45160	GTP-binding protein-like; root hair defective 3 pr	0.05	0.44
AT1G50660	hypothetical protein	0.02	0.44
AT2G45300	5-enolpyruvylshikimate-3-phosphate (EPSP) synthase	0.01	0.44
AT3G45310	cysteine protease-like protein	0.01	0.44
AT5G10760	nucleoid DNA-binding protein cnd41 - like protein	0.03	0.44
AT2G43360	biotin synthase (Bio B)	0.01	0.43
AT1G15740	hypothetical protein	0.02	0.43

AT2G29070	putative ubiquitin fusion-degradation protein	0.01	0.43
AT3G04610	putative RNA-binding protein	0.00	0.43
AT5G66920	pectinesterase like protein	0.00	0.42
AT4G15540	hypothetical protein	0.05	0.42
AT3G14370	putative protein kinase	0.03	0.42
AT2G13820	putative nonspecific lipid-transfer protein precur	0.03	0.42
AT1G29350	unknown protein	0.03	0.42
AT5G65840	unknown protein	0.02	0.42
AT4G11820	hydroxymethylglutaryl-CoA synthase	0.03	0.41
AT5G33320	phosphate/phosphoenolpyruvate translocator precurs	0.03	0.41
AT3G26580	unknown protein	0.03	0.41
AT1G31340	polyubiquitin, putative	0.03	0.41
AT3G08940	putative chlorophyll a/b-binding protein	0.02	0.41
AT5G40710	zinc finger - like protein	0.01	0.40
AT1G78100	unknown protein	0.02	0.40
AT2G23950	putative LRR receptor protein kinase	0.04	0.40
AT1G14010	putative transmembrane protein	0.00	0.40
AT5G05010	coatomer delta subunit (delta-coat protein) (delta	0.02	0.40
AT1G05160	putative cytochrome P450	0.01	0.39
AT2G30040	putative protein kinase	0.03	0.39
AT1G78630	Hypothetical protein	0.04	0.39
AT5G14030	putative protein	0.02	0.39
AT1G52230	photosystem I subunit VI precursor	0.02	0.38
AT3G47520	chloroplast NAD-dependent malate dehydrogenase	0.01	0.38
AT4G35910	putative protein	0.00	0.38
AT1G04250	putative auxin-induced protein, IAA17/AXR3-1	0.05	0.38
AT3G01280	putative porin	0.02	0.37
AT5G47720	acetoacyl-CoA-thiolase	0.04	0.37
AT5G20710	beta-galactosidase	0.03	0.37
AT5G58070	outer membrane lipoprotein - like	0.03	0.36
AT2G24060	putative chloroplast initiation factor 3	0.01	0.35
AT3G02880	putative protein kinase	0.05	0.35
AT1G66430	fructokinase, putative	0.01	0.35
AT3G18440	unknown protein	0.04	0.34
AT2G21790	putative ribonucleoside-diphosphate reductase larg	0.03	0.34
AT2G28000	putative rubisco subunit-binding-protein alpha sub	0.01	0.34
AT5G65260	poly(A)-binding protein II-like	0.03	0.34
AT1G10060	unknown protein	0.00	0.33
AT4G33610	putative protein	0.05	0.33
AT4G29590	putative protein	0.01	0.33
AT4G14230	hypothetical protein	0.01	0.32
AT2G37950	unknown protein	0.03	0.32
AT2G27960	putative cyclin-dependent kinase regulatory subuni	0.01	0.32
AT4G30140	putative protein	0.02	0.31
AT4G25500	splicing factor At-SRp40	0.02	0.31
AT2G01650	unknown protein	0.01	0.31
AT1G47310	unknown protein	0.02	0.31
AT1G45130	putative beta-galactosidase emb|CAB64741.1; simila	0.02	0.31
AT4G11820	hydroxymethylglutaryl-CoA synthase	0.00	0.30
AT1G79560	hypothetical protein	0.00	0.30
AT1G35720	Ca2+-dependent membrane-binding protein annexin	0.03	0.29
AT3G51920	putative calmodulin	0.04	0.28
AT3G09880	B' regulatory subunit of PP2A (AtB'beta)	0.01	0.28
AT5G38420	ribulose bisphosphate carboxylase small chain 2b p	0.01	0.28

AT1G56070	elongation factor, putative	0.00	0.28
AT1G64090	hypothetical protein	0.01	0.28
AT5G52530	putative protein	0.01	0.28
AT3G52090	DNA-directed RNA polymerase II 13.6K chain	0.01	0.27
AT4G15540	hypothetical protein	0.01	0.26
AT2G28570	unknown protein	0.03	0.25
AT3G09260	thioglucosidase 3D precursor	0.04	0.25
AT1G18970	hypothetical protein	0.00	0.25
AT2G22170	unknown protein	0.00	0.22
AT5G49360	xylosidase	0.03	0.17
AT4G00620	putative tetrahydrofolate synthase	0.04	0.15
AT2G47170	ADP-ribosylation factor 1	0.05	0.14

* Gene selected for Northern blotting

7.3 DISCUSSION

From this study it could be shown that information gained from hybridising *Arabidopsis* arrays with heterologous *Aster* cDNAs can identify differentially expressed genes, and may be used directly to characterize transcriptomes in other plant species where genome information is not available or distantly related to *Arabidopsis*. Expression analysis available from extensive *Arabidopsis* microarray databases can provide additional information on signalling pathways for physiological processes that are active in other plants (Horvath et al., 2003). Inspite of the list of genes expressed differentially, one could not be sure until the clone which has been spotted is resequenced and its identity verified in the case of plant species like *Aster* where genome is not yet sequences and very distant to the family of *Arabidopsis thaliana*. But still much of the genes has no direct relation to salt-stress, instead is general, it should be proceeded to know about those genes where a direct role for salt-stress is not established. Importantly noted is the fact that stress originating due to salt, water, drought, flooding were interconnected and act like a chain of stress reactions where salt stress can activate genes also involved in water stress or drought stress, so overlapping of gene expressions in these different kind of abiotic environments results in common set of genes termed as stress induced genes. So, the list of genes in this experiment cannot be ruled out from the other stress than salt stress. This is one of the drawback of microarray technology too. It is still of concern why the Northern blotting has failed, but in time stringency of the hybridisation should be increased and retried again. But this is the possible right step in the right direction because having no available genome information about *Aster tripolium*, it is possible to know the gene expression profile for this aquatic halophyte during salt-stress. In *Mesembryathemum*, major functional genes differentially regulated during salt stress were categorized as cell rescue, defence, aging, water channel, signal transduction. In similarity, H^+-transporting ATPase, putative potassium transport protein, plasma membrane intrinsic

protein were upregulated in response to salt stress in *Aster*. Cysteine synthase gene involved in sulfur metabolic network is down regulated which correlates with reduced enzyme activities of cysteine synthase (refer to chapter 5). One should also keep in mind that the EST collection used for this study comprises only about one-third of the full *Arabidopsis* transcriptome. Also is the fact that approximately half of all the ESTs, CDNAs and genes that have become available are functionally not chracterized, and the frequency of functionally unknown transcripts in cDNA libraries from stressed tissues is even higher (Bohnert et al., 2001). When these unknown functional genesare categorized, probabl our understanding would be become much easier.

Rather than being indicators of stress resistance, many of these response components may be ancillary and irrelevant or may even be indicators of degenerative processes (Ozturk et al., 2002). It is hoped, however, that gene expression arrays will enable the actions of these individual genes to be studied and interpreted in a broader context. Studies such as these would lay the groundwork for mapping regulatory networks and depicting linkages among gene products, biochemistry and whole-plant physiology (Wullschleger and Difazio, 2003). This study in particular will help to identify genes that are regulated during initial salt-stress process in this aquatic halophyte and to understand, network of the complex salt tolerance mechanisms and to know the way halophyte differs from glycophytes in handling salt-stress. Connecting microarray approach alongside the results of physiological aspect and its effect due to salt stress, analysing proton transport through plasma membrane and variations in its protein family, with lipids (sulfolipids) role in salt tolerance will be a great deal of work in *Aster tripolium* for the foreseeable future.

8 GENERAL DISCUSSION

Aster and *Sesuvium* both were able to tolerate seawater salinity level inspite of biomass reduction to a certain extent. Subsequently both *Aster* (3%) and *Sesuvium* (5%) suffered in the growth rate at the highest salt level. Understanding the mechanisms of salt tolerance was the next line of research. *Aster* behaved as a typical halophyte showing high levels of inorganic ion accumulation even at low salinities (Shennan et al., 1987b). Na^+ and K^+ ions were looked at specifically, because if Na^+ is higher than its admissible level it causes metabolic arrest, while K^+ is essential for the proper functioning of many enzymes involved in the metabolism. It has been reported that *Sesuvium*'s growth is stimulated by NaCl of 100-200 mM NaCl in the watering medium but could survive upto 1000 mM NaCl (Mssedi et al., 2000). Na^+ accumulation is two fold higher in *Sesuvium* compared to *Aster* whereas the reverse is true for K^+ accumulation. In *Aster* Na^+ is stored in vacuoles similar to other halophytes to a certain level, but for *Sesuvium* these observations could not be made due to methodological difficulties. To conclude based on Na^+/K^+ accumulation it could be presumed that the succulent *Sesuvium* acts as an includer and *Aster* as excluder. No precise distinction between the salt concentrations tolerated by the resistant plants and by other species is defined, although an arbitrary dividing line is generally drawn at an external concentrations of about 100 mM (Flowers and Läuchli, 1983). Succulent plants normally tend to increase their water capacity to maintain turgor pressure and osmolarity. The argument of Ueda et al. (2003) is that *Aster* has mechanisms to accumulate Na^+ and Cl^- in vacuoles and osmolytes in the cytosol to maintain osmotic potential in the cell. But the above outcome can be compounded by an increase in osmolarity level in both plants with increasing NaCl concentrations, thus balancing the osmotic potential through various compounds (sugars, amino acids, amines etc.), which still needs to be elucidated.

Photosynthetic rates are reduced in both *Aster* and *Sesuvium* at higher NaCl concentrations. CO_2 assimilation rate was severely affected along with depleted CO_2 intercellular concentrations in both *Aster* and *Sesuvium*. *Sesuvium* has high possibilities to be classified as a CAM plant based on the net photosynthesis rate to the concenration of carbondioxide.to the In both plants transpiration has almost ceased with improving water use efficiency. Increased salinity reduces the photosynthetic surface area available for CO_2 assimilation, i.e. that salinity may reduce the expansion of the leaf surface (Papp et al., 1983).

Next attention was focused in the direction of bioenergetics during photosynthesis and membrane transport. The regulation of ions across the plasma membrane (PM) is thought to be achieved by an electrochemical gradient generated by PM H^+-ATPase (Palmgren, 2001).

P-type-H^+-ATPase located in the plasma membrane PM was strongly downregulated from day 3 in *Aster*, but no changes could be observed in *Sesuvium*. The result suggests an alternative way for Na^+ transport in this succulent plant. But published reports cited that salt treatment increased the H^+-pumping capacity of the PM H^+-ATPase in *Atriplex nummularia* roots (Braun et al., 1986) and PM H^+-ATPase was stimulated in *Salicornia bigelovii* Torr. when the plants were grown in media containing 200 mM NaCl (Ayala et al., 1996).

Based on the P-ATPase activity results the interest was to look more closely at this enzyme from *Aster*. *Aster* PM was isolated and the enzyme PM H^+-ATPase was characterized in detail. The purity of the isolated membrane vesicles was shown through inhibition tests with the specific inhibitor Na_3VO_4 and by sequencing the dominant 100 kDa band out from a SDS gel. PM H^+-ATPase activity was at its optimal in the solution having pH 7.0. Optimal concentration was determined to 1 mM. An important investigation was to check the activity in the presence of NaCl *in vitro*. Even at 300 mM NaCl in the assay there was not a trace of an inhibition. Proteins of the PM were separated using two-dimensional electrophoresis to map those highly upregulated or downregulated ones and look for specific candidates in *Aster* during salt tolerance processes. However, little information is available concerning PM proteins (Sussman, 1994), and only recently proteomic data of PM proteins became more abundant (Santoni et al., 1999; Kawamura and Uemura, 2003). But the initial sequencing using *Arabidopsis* genome did not reveal any new or novel genes.

The active transport process is important for the compartmentation of metabolically toxic Na^+ ions inside the vacuole. The tonoplast localized V-ATPase did not show any changes due to NaCl stress in both plants. The main strategy for the salt marsh plant *Suaeda salsa* L. was reported to be the up-regulation of V-type ATPase activity in tonoplast vesicles but by an increase in V-type ATPase protein amount (Wang et al., 2001). Another adaptation to salt might be an increase in the volume of the vacuole due to accumulation of ions and osmolytes (Mimura et al., 2003). The exploration for specially developed or formed storage organs for ions in *Aster* was also investigated using scanning electron microscope (SEM). Although some species have salt glands or can develop succulence to increase their vacuolar capacity for salt accumulation, most halophytes do not have any obvious morphological adaptation (Very et al., 1998). Initial search revealed no peculiar formations when analysed using cryo-preserved leaf samples. Though a more careful approach using transmission electron microscopy (TEM) for analysis in the cell organelles (cell wall and chloroplast) is underway before making any final conclusions about the ultrastructural role in compartmentation of ions.

After knowing the consequences of NaCl stress on P- and V-ATPases the next approach was to look at the energizer of photosynthetic machinery, the F-ATPase, which is structurally similar to V-ATPase. Kanazawa and Kramer (2002) proposed a hypothesis that chloroplast ATP-synthase assumes an important role in regulating adjustments of the photosynthetic system to environmental conditions by varying the relationship of photosynthetic electron transport to Non-photochemical quenching (NPQ). The F-ATPase localized in chloroplast and mitochondria showed a increase in its activity when correlated to NaCl concentrations in the growth medium in *Aster*. In *Sesuvium* no changes could be observed. There is no published report on the F-ATPase activity during salt stress in halophytes for comparison with the results from *Aster* and *Sesuvium*. But exciting to explore is the fact that F-ATPase effects due to NaCl could be related to the increase of sulfolipid in *Aster* and *Sesuvium*. Plnats sulfolipids are known as plant lipids closely associated with F-ATPase of the chloroplast. F-ATPase functioning could be restored only in the presence of sulfolipids (Barber and Gounaris, 1986). Sulfolipid changes due to various abiotic stresses has been documented in glycophytes and it was shown previously that environmental and nutritional factors play a role in the synthesis of sulfoqionovosyl diacylglycerol (SQDG), like water deficit (Müller and Santarius, 1978), temperature (Quartacci et al., 1995; Sato et al., 2003), sulfate deprivation (DeKok et al., 1997), and phosphate starvation (Essigmann et al., 1998; Yu et al., 2002), but concrete evidence for a role of SQDG in halophytes was still missing dring salt stress. Both F-ATPase and sulfolipids increased strongly in relation to high NaCl in *Aster* and *Sesuvium*. To confirm the results similar investigation was carried out in *Thellungiella halophila*, a salt tolerant relative of *Arabidopsis* and in *Arabidopsis* itself. In *Thellungiella* the sulfolipid content increased due to the effect of NaCl echoing the result of *Aster* and *Sesuvium*, but no significant changes could be observed in *Arabidopsis*. These results indicate that sulfolipids might play a role in salt tolerance mechanisms in halophytes. When looked at the fatty acids profile of sulfolipids one has to keep in mind that the membrane fluidity changes in both *Aster* and *Sesuvium*, it is astonishing to note that the unsaturation of fatty acids were decreasing with increasing NaCl concentrations and experimental period in *Aster*, but no changes could be observed in *Sesuvium*. The LC-MS analysis of sulfolipids at the level of theirfatty acid composition pointed to the direction of a novel sulfolipid molecule similar in both *Aster* and *Sesuvium*. In spite of similar sulfolipid molecules, both plants have its own way of the compound's utilization.

Aster was chosen for further analysis of the sulfur metabolism in high salt conditions. Sulfate availability in seawater is about 200-fold higher than in fresh water ecosystems (Oren,

2002). The effect of the enzymes O-acetyl-L-serine(thiol) lyase (OAS-TL) and β-cyanoalanine synthase (CAS) in these halophytes under salt stress was investigated. The experiments showed that the sequence of sulfate reduction and cysteine metabolism was severely affected. The enzyme activities were more affected in roots than in the leaves of salt treated plants. Analysis of the regulation of the metabolic network of sulfur compounds in plants is complex due to the presence of multi-gene families at either step and to the compartmentation of the pathways in the cell (Droux, 2003). The outcome of these experiments demonstrate the impairment of sulfur metabolism in salt stress.

Further experiments were designed to look at the pigmental variations due to NaCl treatment in *Aster* and *Sesuvium* after knowing that the photosynthetic rate was adversely affected. Chlorophyll plays a major role in the photosynthetic reactions and also has been hypothesized to be associated with sulfolipids. Carotenoids are part of the antennae complex and protect the reaction center from the excess electrons through xanthophyll cycling. Chlorophyll contents increased in *Aster* whereas no significant changes were reflected in *Sesuvium* under varying NaCl concentrations. Though a small increase of carotenoid contents in *Aster*, their total amount seemed to be unaffected by the short-term NaCl effect.

No investigation into the photosynthetic perfomance of plants under field conditions seems complete without some fluorescence data (Maxwell and Johnson, 2000). Chlorophyll fluorescence has been shown to be a non-invasive, powerful, and reliable method for assessing PSII function (Lu *et al*., 2003). The maximum and actual yield has no significant changes with increasing salt concentrations. But important to note is that maximum yield of *Aster* and *Sesuvium* is around 0.80 relative units which is an indicator of a metabolically active plant. The maximum electron transport rate also showed no significant changes. But an important finding might be the changes in the NPQ due to salt stress in both *Aste*r and *Sesuvium*. Previous studies have shown that salt stress induced photodamage to PSII when salt-stressed plants were exposed to high light (Mishra et al., 1991; Masojidek and Hall, 1992; Belkhodja et al., 1994). The changes in the NPQ vary between *Aster* and *Sesuvium*, but the changes due to the effect of NaCl and combinatory affect with light stress looked similar. The NPQ value in *Sesuvium* is almost half that of *Aster* implying different strategies employed. Initial hypothesis might be that *Sesuvium* needs less energy and emits less heat compared to *Aster*. Composition of carotenoids and the xanthophylls cycle analysis is very important to understand the above proposed hypothesis in *Aster* and *Sesuvium*.

After observing at the angle of physiology, biochemistry and proteomics, it is worthwhile to look at changes at the molecular level due to salt stress. As a pilot study it was tried with

Aster by using the microarray technology. The *Aster* genome is not yet sequenced. The *Arabidopsis* expressed sequenced tags (EST) clones were hybridised with labelled RNA from *Aster* to investgate the whole transcriptome variation due to salt stress. This powerful method could be used to analyse in mass metabolically affected genes and pick them up for further study in detail. Initial data obtained were not enough for any final conclusion. The approach is continuing to try modified conditions that yields more reproducible microarray data. Heterologous microarray hybridisation using *Arabidopsis* EST collections can be successfully used.

The development of halophytes into cultivated plants represents a biological solution to agricultural salinity problems and an alternative approach to expand cultivation onto unfavourable land (O'Leary, 1984). But halophytes in general have varying levels of salt tolerance and growth conditions. Hence, it is essential to pick out the right halophytes which are suitable for human needs. Understanding the basics of salt tolerance could provide clues for strategies to improve salt tolerance of conventional crops as well as to predict ecological and agronomic adaptations of halophytes (Glenn et al., 1994). One emphasis of this study was to optimise the growth conditions for the better productivity of halophytes *Aster* and *Sesuvium* which could become a major agricultural product for human beings and cattle in the near future. The importance of this investigation is to grow *Aster* and *Sesuvium* in the salt-affected soils of the arid regions and irrigate them with seawater where freshwater is not available or not in surplus. As such this approach would be a multi-purpose one by reusing the salinized land, high nutrient supplement for human beings, provide low-cost fodder for the cattle, improve the economical status for the farmers in particular along the coastlands, landscaping and dune stabilization. This study comes out with more than one probable conclusions and recommendations for the growth of *Aster* and *Sesuvium* in the highly salinized soil which could not be otherwise used for agricultural purposes.

To start with both halophytes *Aster* and *Sesuvium* could be used as model plants for the future ecophysiological and biochemical investigations and in parts for molecular approaches carried out under saline conditions. These halophytes could be grown in little less than half-strength seawater (1.5% NaCl) (*Aster*) and seawater (*Sesuvium*) concentrations, conveniently in the green house with good production of biomass which is essential for any physiological investigations. Because *Aster* has its growth limited when used close to seawater concentration (3%) whereas growth was affected in *Sesuvium* only at the 5% NaCl level. Leaves of both the plants impart a peculiar salty taste along with highly nutritive osmoprotectants which were made of proteins, amino acids, sugars, and amines. In addition

both plants have more than 90% leaf water content and certain amount of NaCl which would make them better fodder crop for the live stock population. Very important is the ease at which both plants can be grown either in the field or in the nursery. *Aster* for instance can be seed propagated and seeds are produced annually in thousands, whereas *Sesuvium* biomass production is much easier with cuttings from the existing shoots. This significantly reduces the cost of buying seeds for the rural farmers. *Sesuvium* in particular would be very useful when grown along the shores both for beautification without much expense and also as sand-binder utilizing its creeping nature of growth. *Aster* could be used as an alternative to spinach in temperate regions which would serve as a fibre rich food essentially needed for human system daily functions.

Inspite of a very positive outlook generated out of this investigation, still more facts have to be confirmed for a much better prospects in future. Further *Aster* and *Sesuvium* could be used as a model halophytes for the molecular approaches by constructing cDNA libraries, cloning sulfolipids biosynthesis genes, P-ATPase genes and analysing their expressions in various simulated experimental conditions having high salt and finally to be able to establish a transformation system for both the plants. This would enhance the understanding of halophyte salt tolerance mechanisms at the molecular level.

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List of publications

Published

Balasubramanian R, Schmidt A, Papenbrock J (2001) Investigation of sulfolipid contents and their rate of biosynthesis in halophytes treated with different salt concentrations. In: Plant nutrition – Food security and sustainability of agro-ecosystems. Horst WJ et al. (eds) Kluwer Academic Publishers, Dodrecht, pp 434-435

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In Press

Balasubramanian R, Papenbrock J, Schmidt A (2003) Connecting sulfur metabolism and salt tolerance mechanisms in the halophytes *Aster tripolium* and *Sesuvium portulacastrum*. *Trop. Ecol.,* in press. (**Chapter 5**)

In preparation

Balasubramanian R, Debez A, Reeck T, Stelzer R, Huchzermeyer B, Schmidt A, Papenbrock J. *Aster tripolium* and *Sesuvium portulacastrum*: Two halophytes, two different strategies in salt tolerance. (**Chapter 3**)

Balasubramanian, R, Zorn H, Papenbrock J. Analysis of sulfolipid contents from halophytes and glycophytes. (**Chapter 4**)

Balasubramanian R, Hellmer J, Päatzold R, Stelzer R, Lemaitre-Ggullier C, Van Dorsselaer A, Papenbrock J. Characterization of the plasma membrane H^+-ATPase of the halophyte plant *Aster tripolium*. (**Chapter 6**)

Balasubramanian R, Papenbrock J, Höfgen R, Nikiforova V. Results of heterologous microarray analysis: Hybridization of *Arabidopsis thaliana* filters with *Aster tripolium* probes. (**Chapter 7**)

Erklärung

Ich versichere, dass die Dissertation selbständig verfasst und die benutzten Hilfsmittel und Quellen, sowie gegebenenfalls die zu Hilfsleistungen herangezogenen Institutionen, vollständig angegeben wurden und die Dissertation nicht schon als Diplomarbeit oder ähnliche Prüfungsarbeit verwendet worden ist.

Hannover, den 05. Januar 2004 Balasubramanian Ramani

Grateful I am….

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SAGENHAFT!!!!

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