

Biochemical and physiological mechanisms leading to salt tolerance

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Abstract: We showed that malic enzyme and F-type ATPase isolated from glycophytes and halophytes do not differ significantly in their amino acid composition. Isolated enzymes from both types of plants showed nearly identical salt sensitivity, each, but enzyme activities in fresh homogenates from halophyte leaves apparently were more salt resistant as compared to the ones from glycophytes. These results suggest that some cytosolic compounds other than the tested enzymes bring about salt tolerance. We observed distinct differences in photosynthate export out of the chloroplasts when comparing salt stress response of glycophytes and halophytes. In contrast to glycophytes metabolite leakage out of the chloroplasts or internal metabolite pool size are more strictly regulated in halophytes. Pronounced differences were observed, when measuring P-type ATPase activities at the plasmalemma. Apparently, most of the difference between salt tolerant plants (halophytes) and glycophytes is due to a faster and more effective response of the former bringing about ion homeostasis by adaptation of transmembrane transport to saline conditions and coordination of metabolism.

Resumen: Nosotros mostramos que la enzima málica y la ATPasa de tipo F aisladas de glicófitas y halófitas no difieren significativamente en su composición de aminoácidos. Las enzimas aisladas a partir de ambos tipos de plantas mostraron sensibilidades casi idénticas a la sal, pero las actividades enzimáticas en homogenados frescos de hojas de halófitas aparentemente fueron más resistentes a la sal en comparación con los de las glicófitas. Estos resultados sugieren que algunos compuestos citosólicos diferentes de las enzimas ensayadas son los responsables de la tolerancia a la sal. Observamos diferencias claras en la exportación de fotosintatos desde los cloroplastos al comparar las respuestas al estrés salino de las glicófitas y las halófitas. La fuga de metabolitos glicófitos de los cloroplasto o el tamaño de las reservas internas de metabolitos están regulados más estrictamente en las halófitas que en las glicófitas. Se observaron diferencias marcadas en la medición de las actividades de la ATPasa de tipo P en el plasmalema. Aparentemente, la mayor parte de la diferencia entre las plantas tolerantes a la sal (halófitas) y las glicófitas se debe a una respuesta más rápida y más efectiva entre las primeras, lo que se traduce en una homeostasis iónica por medio de la adaptación a condiciones salinas del transporte transmembranal, y en la coordinación del metabolismo.

Resumo: Mostrou-se que a enzima málico e o isolado do tipo F ATPase de glicófitas e halófitas não diferem significativamente na composição dos seus aminoácidos. Os enzimas isolados dos dois tipos de plantas mostraram, em cada uma, uma sensibilidade quase idêntica ao sal mas as atividades enzimáticas em homogeneizados frescos de folhas halófitas eram aparentemente mais resistentes ao sal quando comparadas com as provenientes de glicófitas. Estes resultados sugerem que certos compostos citosólicos, além das enzimas testadas conferem tolerância ao sal. Observámos diferenças distintas na exportação de fotosintetizados dos cloroplastos quando se compara a resposta de espécies glicófitas e halófitas ao stress salino. Em contraste com as glicófitas, o escoamento de metabolitos dos cloroplastos ou a dimensão do

reservatório interno de metabolitos é mais estritamente regulado nas halófitas. Observam-se diferenças pronunciadas, quando medindo as actividades das ATPases do tipo P, no plasmalema. Aparentemente, muitas das diferenças entre espécies tolerantes ao sal (halófitas) e glicófitas é devido a uma resposta mais rápida e mais efectiva da primeira conduzindo à homeostase iónica por adaptação do transporte transmembranar às condições salinas e à coordenação do metabolismo.

Key words: *Aster tripolium*, ATPase, halophytes, ion homeostasis, NaCl salinity, salinity tolerance, salt stress, salt tolerance, stress response.

Introduction

Soil salinity is a major abiotic stress for plant agriculture. Sodium ions in saline soils are toxic to plants because of their adverse effects on K⁺ nutrition, cytosolic enzyme activities, photosynthesis, and metabolism (Niu *et al.* 1995). To date about one third of the arable land under irrigation is salt-contaminated at an extent limiting its agricultural use. Therefore, the UN have called in their 2001 report for molecular engineering of crops in order to produce seeds for countries suffering from the lack of water, salt contamination of soils, and desertification. In plant breeding, the standard approach to this problem would be to increase salt tolerance of conventional crop plants, but with cereals, for instance, the grain yield is generally low. The alternative approach is to make use of the plants that already have the requisite level of salt tolerance, and are highly productive at high external salinity levels: the halophytes. A pre-requisite of any breeding project is to identify at molecular level the mechanism bringing about salt tolerance.

Comparative physiological and biochemical studies of glycophytic crops and halophytes have led to the conclusion that genetic information for salt stress perception and tolerance exist in all higher plants (Amzallag *et al.* 1990; Lee *et al.* 1999). But the finding that halophytes seem to lack unique mechanisms leading to salt tolerance complicates the task of breeding salt-tolerant crops. Obviously the basic requirement for survival under salt stress are (i) osmotic adjustment as well as (ii) compartmentation bringing about partition of toxic ions away from the cytosolic compartment, by this allowing to use NaCl as “cheep osmolytes” in addition to organic compounds. Both reactions

are energy-consuming and directly or indirectly compete with other metabolic reactions for photosynthates (Apse *et al.* 1999; Binzel *et al.* 1988; Glen *et al.* 1999; Hajibagheri *et al.* 1987; Niu *et al.* 1995; Storey *et al.* 1983; Yeo 1998). This latter interpretation agrees with the observation that growth is less retarded upon salt shock of halophytes as compared to glycophytes (Adams *et al.* 1992; Glen *et al.* 1999; Greenway & Munns 1980; Yeo 1998). On the other hand, as surplus salt is stored in the vacuoles, plant growth, or more precisely the permanent production of new vacuoles, is a major factor limiting salt tolerance (Lerner 1999). A basic difference between halophytes and glycophytes is the capacity of the former to survive salt shock, which may occur with tidal and rainfall events. This greater capacity allows halophytes to more readily establish metabolic steady state for growth in a saline environment (Braun *et al.* 1986; Casas *et al.* 1991; Hassidim *et al.* 1990; Niu *et al.* 1993).

Ion homeostasis is a pre-requisite of plant growth. In saline environment it depends on the capacities of membrane transport systems, ATPases, secondary active transporters and channels being the main components to be mentioned (Niu *et al.* 1995; Sze & Palmgren 1999). Most of these proteins known to date were identified from structure/function information or by functional complementation of transport-deficient mutants (Dreyer *et al.* 1999). It is well documented in the literature that increased ATPase-mediated transmembrane proton transport is an early plant cell response to salt stress (Braun *et al.* 1986; Koyro *et al.* 1993; Lüttge & Ratajczak 1997; Ratajczak *et al.* 1994; Sze & Palmgren 1999; Watad *et al.* 1991; Zhien *et al.* 1997). This increased activity has been attributed to greater protein abundance (Ratajczak

et al. 1995), changes in kinetic properties (Reuveni *et al.* 1990), differential subunit composition and transcriptional regulation of ATPase subpopulations (Binzel 1995; Narasimhan *et al.* 1991).

The analysis of plant osmolytes produced upon salt stress is among the points of special interest of researchers. Osmotic pressure and turgor are quickly regulated by modifying ion fluxes. But, over a longer period of time osmolyte synthesis comes into play controlling ion homeostasis at the level of cell membranes and multienzyme complexes (Bray 1997; Messedi *et al.* 2000). It is generally agreed that osmolytes (i) do not inhibit normal metabolic reactions, (ii) are accumulated in the cytosol upon changes of external osmolarity and (iii) are capable of protecting structures and osmotic balances (Delauney & Verma 1993; Ford 1984; Yancey *et al.* 1982). Osmolytes are typically hydrophilic and are thought to replace water at the surface of proteins and membranes, thus acting as osmoprotectants, reducing inhibitory effects of ions (Nelson *et al.* 1998; Solomon *et al.* 1994; Yeo 1998). With respect to this interpretation the local concentration of osmoprotectants will be more important than the overall cellular concentration, a fact not easy to be experimentally approached (Jolivet *et al.* 1982; Zhao *et al.* 1992). Another explanation of osmolyte function is that they may act as sinks for reducing power. This may be an important function subsequent to metabolic disturbance brought about by salt stress (Bohnert *et al.* 1995; Nuccio *et al.* 1999; Zhu *et al.* 1997). In most cases, osmolyte synthesis is directly connected to basic metabolism (McCue & Hanson 1990; Nuccio *et al.* 1999). It has been found that the enzymes required for osmolyte synthesis from primary metabolites are stress-induced (Adams *et al.* 1992; Azaizeh & Steudle 1991; Ishitani *et al.* 1996; Morgan & Drew 1997; Nelson *et al.* 1998; Yoshihara *et al.* 1997; Zhang *et al.* 1995). From this it is clear that osmolytes compete with plant metabolic pathways for their substrate. Osmolyte synthesis can hinder plant productivity, growth and the import into fruits and other storage organs of secondary metabolites. This may be an important side effect to be monitored in breeding projects of salt-tolerant crops.

In this paper, we have re-investigated salt effects on primary reactions of photosynthesis: light reactions, enzyme activities and photosynthate transport, as well as ATPase activities. We show

that monitoring such effects subsequent to salt stress application can offer insight into regulation mechanisms resulting in salt tolerance. Possible interactions between cellular metabolism and energy status are discussed.

Materials and methods

Plants were grown in a turf-soil mixture (Fruhstorfer Erde) in our greenhouse at 28-32 °C (14 hours day) and 18-22 °C (10 hours night) using additional illumination of Philips/Norka 160 white light lamps (198,4 $\mu\text{mol m}^{-2} \text{s}^{-1}$, Quantum Sensor, Skye Instruments, Wales). The substrate was watered twice a day using tap water. For salt stress experiments the soil had to be pre-treated for 8 days by watering at the indicated NaCl concentration prior to planting the experimental plants. – All plants were germinated and subsequently grown for two weeks in untreated soil prior to subjection to experiments. For the preparation of cell homogenates leaves were harvested, frozen in liquid nitrogen and ground. The frozen powder was kept under liquid nitrogen until it was rapidly thawed by mixing with an isolation medium. The standard incubation mixture consisted of: 300mM sucrose, 50 mM HEPES buffer, pH 8.0, 2 mM MgCl_2 20 mM NaCl, 2 mM EDTA, 50 μM polyvinylpyrrolidone, 0.1% (w/v) BSA, 1 mM DTE, and 5% (w/v) frozen leaves powder. The mixture was filtered through 30 μm Monylgaze (Zuercher Beuteltuchfabrik, Rueschlikon), and then centrifuged at 1.000 x g for 5 minutes. The green and slightly turbid supernatant was immediately used for enzyme assays.

For chloroplast preparation two different isolation mixtures had to be subsequently used. The first one contained 300 mM sorbitol, 50 mM MES buffer, pH 6.5, 2 mM MgCl_2 , 1 mM MnCl_2 , 30 mM NaCl, 0.5 mM KH_2PO_4 , 5 mM ascorbate, 4 mM cysteine, 2 mM EDTA, 50 μM polyvinylpyrrolidone, and 5% (w/v) fresh leaves. This mixture was homogenized 5 times for 3 seconds each in a Waring blender and then filtered through 30 μm Monylgaze. After 2 minutes of centrifugation at 1.500 x g the sediment was re-suspended in a second medium consisting of: 300 mM sorbitol, 50 mM HEPES buffer, pH 7.8, 2 mM MgCl_2 , 1 mM MnCl_2 , 30 mM NaCl, 0.5 mM KH_2PO_4 , 2 mM NaNO_3 , 2 mM EDTA, 200 mM choline chloride, and chloro-

plasts corresponding to 0.1 to 0.15 mg chlorophyll per ml. – Chlorophyll was determined after Arnon (1949). After another centrifugation the sediment was re-suspended in this second medium, the chlorophyll concentration was adjusted to 2.5 mg ml⁻¹ and this chloroplast suspension was stored in the dark in an ice bath until use. This standard protocol had to be modified in some experiments. Phosphate had to be omitted from the media, for instance, when we intended to subsequently measure ATPase activity. ATPase activities in cell homogenates were measured using the method of Koyro *et al.* (1993). The standard incubation mixture contained 50 mM of the appropriate buffer (pH 6.0 to 7.0: MES; pH 6.5 to 8.5: TES; pH 7.5 to 9.0: HEPES), 5 mM MgCl₂, 50 mM KCl, 400 mM sucrose, 0.5 mM [γ -³²P] ATP, and sample material corresponding to 0.1 mg of protein per ml. Protein concentration was determined after Bradford (1976). The incubation was performed in a water bath at 30°C and the reaction was stopped by addition of HClO₄ to a final concentration of 0.3 M.

Enzyme activities were measured by monitoring absorption changes at 340 nm in a double-beam photometer (Kontron Uvicon 930) as described previously (Huchzermeyer 1989; Paquet-Durant 1998). Proteins have been hydrolyzed using the method described by Stahl (1967): Per 50 μ g of purified protein, 1g Amberlite IR-112 (Serva, Heidelberg) and 5ml 80% (v/v) ethanol were mixed and sealed under nitrogen gas in a glass tube. The tube was placed in a water bath at 95°C for 12 hours. Afterwards the amino acids have been extracted from the ion exchanger by three subsequent washes with 10% (w/v) ammonium hydroxide. The samples were lyophilized before subsequent amino acid analysis using the method described by Carlton & Morgan (1989). The 9-fluorenylmethylchorismate-derivatized amino acids were separated by reversed phase HPLC using a Beckman 110 system.

Results and discussion

It has been published that enzymes isolated from Halobacteria differ in their amino acid patterns from those of Eubacteria (Lanyi 1974). In Halobacteria an enhanced proportion of acidic amino acid residues has been found (Lanyi 1974). It is argued that salt tolerance of these organisms is based on this difference. Moreover, the enzymes

depend on an increased salt concentration to compensate for intrinsic electronegativity of the proteins (Dundas 1977; Lanyi 1974). With respect to these findings we have checked, whether salt tolerance of halophytes may be brought about by modified amino acid patterns of relevant enzymes as well. We have compared amino acid patterns of enzymes isolated from glycophytes and halophytes, respectively. Some of these enzymes, like chloroplast ATPase from both species *Pisum sativum* and *Aster tripolium*, appeared to be salt tolerant, while the catalytic activities of other enzymes, pyruvate kinase (data not shown) and malic enzyme for instance, were inhibited by 150 mM added NaCl (Fig. 1). Nevertheless, we did not find any significant difference in the amino acid composition of isolated enzymes. Though there was no clear separation achieved of methionine and valin as well as isoleucine, leucine and phenylalanine, our data allow excluding that salt tolerance is brought about by a variation of amino acid patterns. Glutamic acid (pyruvate kinase: *Pisum sativum* – 10.6%; *Aster tripolium* – 10.4% of total amino acids) and aspartic acid (pyruvate kinase: *Pisum sativum* – 4.7%; *Aster tripolium* – 4.7% of total amino acids) clearly could be separated and these acidic amino acid residues have been found to be most important to bring about salt tolerance of halobacteria (Lanyi 1974). With respect to these findings it has to be concluded that factors other than amino acid patterns of enzymes under investigation are controlling salt tolerance.

In further experiments we have measured catalytic activities of relevant enzymes both, in crude extracts and after isolation of the respective enzyme. In Fig. 1 a set of experimental data is shown for reference. It is obvious that there is no significant difference in salt effects on ATPase activities isolated from glycophytes and halophytes, respectively. Some other enzymes appeared to be salt sensitive, because a clear reduction of their catalytic activity was found in the presence of 150 mM added NaCl. But we had to learn that isolated enzymes from both plant types behaved quite similar: With stepwise purification enzymes from halophytes lost their salt tolerance more and more and with respect to salt sensitivity, resembled enzymes prepared from glycophytic plants (Fig. 1). These results correspond to the above-mentioned data on amino acid patterns. Both suggest that salt tolerance is caused by ef-

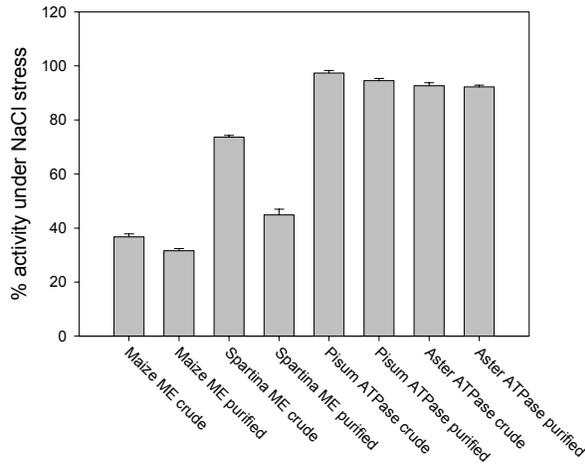


Fig. 1. NaCl inhibition of enzyme activities. Enzyme activities have been measured either in crude leaf extracts or after ammonium sulfate precipitation and Sephadex gel permeation chromatography (“purified” enzyme). Enzyme “purification” was performed in order to remove non-protein compounds (sugars etc.). Results from 5 repeats performed on different days in May are averaged. Salt induced inhibition of *in vitro* enzyme assays is shown for two enzymes differing in their salt sensitivity. Salt treated malic enzyme (“ME”) has been incubated in the presence of 150 mM NaCl added to the standard incubation mixture. Enzyme activities of the controls were: 16.47 $\mu\text{mol NADH} \cdot (\text{h} \cdot \text{mg protein})^{-1}$ [maize, “purified”], 2.22 $\mu\text{mol NADH} \cdot (\text{h} \cdot \text{mg protein})^{-1}$ [maize, crude extract], 14.36 $\mu\text{mol NADH} \cdot (\text{h} \cdot \text{mg protein})^{-1}$ [*Spartina*, “purified”], and 1.87 $\mu\text{mol NADH} \cdot (\text{h} \cdot \text{mg protein})^{-1}$ [*Spartina*, crude extract]. F-type ATPase, as an example for a salt tolerant enzyme, has been isolated from *Pisum sativum* and *Aster tripolium* leaves. Catalytic activities were measured in the absence (control) and presence of 500 mM added NaCl. Control rates were: 68.18 $\mu\text{mol ATP hydrolyzed} / (\text{h} \cdot \text{mg chlorophyll})^{-1}$ [*Pisum sativum*, crude extract], 270.67 $\mu\text{mol ATP hydrolyzed} / (\text{h} \cdot \text{mg chlorophyll})^{-1}$ [*Pisum sativum*, crude extract], 149.56 $\mu\text{mol ATP hydrolyzed} / (\text{h} \cdot \text{mg chlorophyll})^{-1}$ [*Aster tripolium*, “purified” enzyme], and 629.65 $\mu\text{mol ATP hydrolyzed} / (\text{h} \cdot \text{mg chlorophyll})^{-1}$ [*Aster tripolium*, “purified” enzyme].

fects other than the primary enzyme structure. We should rather look for some other cytosolic components interacting with the enzymes.

Physiological studies, chlorophyll fluorescence and gas exchange measurements, for instance, indicate that glycophytes and halophytes differ in their capacity to adapt basic reactions of photosynthesis to their needs under stress (Genty & Meyer

1994; Imai & Murata 1979; Kumar *et al.* 2000; Trossat *et al.* 1998). In earlier experiments we have measured salt effects on primary reactions of photosynthesis at sub-cellular level by measuring photosynthetic electron transport, building the proton motive force at the thylakoid membranes, and photophosphorylation (Huchzermeyer & Heins 2000). With NaCl concentrations below 200mM in the incubation mixture there was no significant inhibition of any of these reactions, irrespective of the experimental plant used. In the literature on plant stress response such high cytosolic NaCl concentration have not been reported yet. Plant salt tolerance and sensitivity, respectively, therefore, cannot be related to NaCl effects on any of these three primary reactions.

From experiments with herbicides it is well documented that there is a strict correlation among partial reactions of photosynthesis. Any adverse condition inhibiting the photosynthetic electron transport rate or Calvin cycle enzymes can have a feedback effect on primary photosynthetic events and cause chlorophyll fluorescence to increase (Genty & Meyer 1994; Müller *et al.* 2001). We, therefore, continued our search and measured salt effects on the export of photosynthate out of intact chloroplasts. As shown in Fig. 2, we used an indirect approach of this problem and measured light induced oxygen production of intact chloroplasts prepared from glycophytes and halophytes, respectively. In such an experiment at the dark to light transition the initial rate of oxygen production is hampered if chloroplasts possess a high photosynthate export capacity. This effect can be explained in terms of metabolite leakage resulting in sub-optimal concentrations of intermediates of photosynthesis inside the chloroplasts. Therefore, a high export rate of intermediates of the Calvin cycle in the dark period prior to the illumination will result in low rates of subsequent light induced oxygen production. In order to find hints to identify the system responsible for the leakage of intermediates, we added substrates for antiporters during the dark incubation step. The data shown in Fig. 2 indicate that Mg^{2+} and phosphate, which are cofactors and substrates, respectively, of the chloroplast phosphate translocator, stimulate the leakage of the Calvin cycle intermediate. When comparing our experimental conditions to those described in the literature, it looks reasonable that the phosphate translocator is involved in this pho-

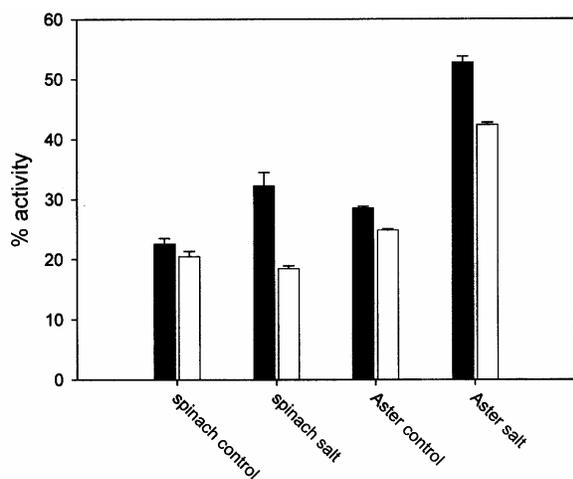


Fig. 2. Photosynthetic electron transport. Upon illumination in a Clark type oxygen electrode, initial rates of oxygen evolution by chloroplasts isolated from spinach and *Aster tripolium* have been measured in the presence of 2 mM phosphate (black bars) and 0.5 mM MgCl₂ (open bars) in the incubation mixture. Where indicated ("salt") the plants were grown in the presence of 200 mM NaCl. Oxygen evolution rates of the controls were: 98.5 $\mu\text{mol O}_2 (\text{h} * \text{mg chl.})^{-1}$ (spinach); 98.5 $\mu\text{mol O}_2 (\text{h} * \text{mg chl.})^{-1}$ (spinach + salt); 88.0 $\mu\text{mol O}_2 (\text{h} * \text{mg chl.})^{-1}$ (*Aster*); 91.1 $\mu\text{mol O}_2 (\text{h} * \text{mg chl.})^{-1}$ (*Aster* + salt).

tosynthate leakage. (Cséke & Buchanan 1986; Flügge & Heldt 1977; Flügge *et al.* 1983; Underwood & Gould 1980).

Comparing data from experiments with glycophytes (spinach) and halophytes (*Aster tripolium*), respectively, some typical salt stress effects can be identified: Chloroplasts isolated from salt stressed and non-stressed glycophytes showed minor differences in substrate leakage. Both preparations resembled chloroplasts isolated from non-stressed halophytes. When 2 mM phosphate was added to the incubation medium in the dark, electron transport rates in glycophytic chloroplasts from plants grown under salt stress were significantly less inhibited in a subsequent illumination period, as compared to preparations from control plants (Fig. 2). Therefore, it may be concluded that phosphate or phosphorylated sugars are among the compounds limiting photosynthesis of isolated chloroplasts. Obviously they are leaking out of the chloroplasts, resulting in intrinsic substrate concentrations below the K_m values of their specific enzymes. From earlier experiments (Bickel &

Schultz 1976) we knew that after a dark incubation period it takes 5 to 15 minutes of illumination to re-build physiological intermediate concentrations within the chloroplast stroma. It has to be pointed out that a reduced photosynthetic capacity during the first 15 minutes of illumination in the morning is a feature of minor importance in vivo. Our experimental approach is aimed at introducing an indirect measure for uncontrolled substrate leakage out of the chloroplast. With this background information in mind our results indicate that spinach like other glycophytes has some capacity to control upon salt stress the leakage of phosphorylated compounds out of the chloroplasts. But, looking at the data from experiments with *Aster tripolium* (Fig. 2) it becomes obvious that this capacity is much more pronounced in halophytes. As compared to the untreated control plants, preparations from salt stressed halophytes showed enhanced rates of oxygen production both, in the presence of external MgCl₂ and added phosphate. There are two possible explanations for this observation: (i) Plant salt stress results in a reduced photosynthate export out of chloroplasts in the dark. (ii) Chloroplasts from salt stressed plants possess an enhanced internal pool size of photosynthates. – Currently we are not in the position to exclude one of these possibilities.

As already pointed out, incoming salt has to be excluded from the cytosol in order to prevent adverse effects on enzymes. Active transport will take place at the expense of the cytosolic energy status and this way may interfere with plant growth. In order to investigate this problem in more detail, we have monitored P-type ATPase activities of glycophytes and halophytes after salt exposure. The result of such an experiment is shown in Fig. 3. Though ATPase activity was somewhat higher in *Aster tripolium* (82.5 $\mu\text{mol ATP hydrolyzed} * \text{mg protein}^{-1} * \text{h}^{-1}$) as compared to spinach (45.0 $\mu\text{mol ATP hydrolyzed} * \text{mg protein}^{-1} * \text{h}^{-1}$), activities from glycophytes and halophytes resembled each other with respect to substrate- and pH-dependencies.

Subsequent to salt stress ATPase activities were stimulated in both plant types, and again, highest specific activities were found with preparations from halophytes (Measurement after two days of salt stress: *Aster tripolium*, 110.5 $\mu\text{mol ATP hydrolyzed} * \text{mg protein}^{-1} * \text{h}^{-1}$; *Spinacia oleracea*, 66.7 $\mu\text{mol ATP hydrolyzed} * \text{mg protein}^{-1} * \text{h}^{-1}$).

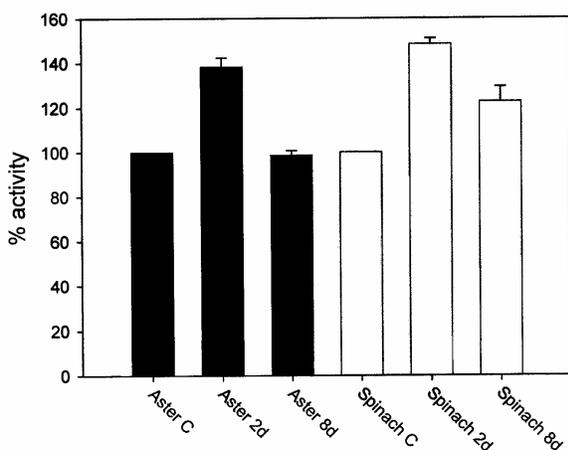


Fig. 3. P-type ATPase. P-type ATPase activities have been measured in extracts from spinach and *Aster tripolium* leaves. Three batches of both plant species were analyzed: (C) control plants, (2d) leaves taken from plants subsequent to 2 days of salt stress (200mM NaCl), and (8d) leaves taken from plants treated 8 days with 200 mM NaCl in the growth medium. ATPase activities measured in preparations from the control plants were $45.0 \mu\text{mol ATP hydrolyzed (h} \cdot \text{mg chl.)}^{-1}$ and $82.5 \mu\text{mol ATP hydrolyzed (h} \cdot \text{mg chl.)}^{-1}$ for spinach and *Aster*, respectively.

h^{-1}). A decay with time of the enhanced activities was observed, provided the experimental plants could tolerate the salt stress. In our experiments the most significant difference between halophytes and glycophytes was the capacity of the former to faster adapt to control rates of ATPase activity subsequent to salt stress (Fig. 3).

In summary, we can conclude that we have observed in our experiments two main differences between glycophytes and halophytes. The transport systems for intermediates of photosynthesis of the chloroplast envelope membranes become changed upon salt stress resulting in an enhanced pool size of intermediates subsequent to a period of dark incubation. It may be speculated that this allows both, a more precise control of metabolite transport across the chloroplast envelope membranes, and a more effective photosynthesis. Moreover, it has to be kept in mind that chloroplasts run the risk of producing reactive oxygen species in the light, if they run out of acceptors for electrons. It has been shown that salt treatment of plants triggers oxidative defence mechanisms to compensate for oxidative stress (Savouré *et al.*

1999 and citations therein). Depending on the plant species different radical scavengers like carotenoids, glutathione, ascorbic acid etc. can be detected. The activation of Cat genes apparently is among the response reactions of all salt stressed plant species. But halophytes, as compared to glycophytes, appear to be capable of a faster adaptive response. This interpretation is in line with the finding (Gupta *et al.* 1993) that salt tolerance of some plant species can be improved by overexpression of enzymes increasing the intrinsic pool size of plastidic radical scavengers.

The improved potential of halophytes to adjust P-type ATPase activity within a short period of time subsequent to a salt stress event to the control rate prior to the stress is an important finding (Fig. 3). This result indicates that halophytes, better than glycophytes, are capable of returning to ion homeostasis of their cells. This allows halophytes faster than glycophytes to return to normal cytosolic phosphorylation potential, metabolism, and growth rate. This is essential with respect to a pre-requisite of salt tolerance already pointed out in the introduction: One aspect to obtain salt tolerance is to sequester surplus salt into the vacuoles. As vacuoles have a limited capacity to hold salt, this interpretation implies that permanent growth and permanent production of new vacuoles is the basis of salt tolerance. Our preliminary measurements of V-type ATPase activity apparently support this interpretation (data not shown). There is a strong correlation between P-type and V-type ATPase activities. But we had to learn that there is a much higher variability among individual plants with respect to V-type ATPase rather than P-type ATPase activity. We currently are repeating our tests to have more reliable results. Another question is to understand the reason for the high variability of plant material resulting in a poor reliability of our current experimental data on V-type ATPase activity.

It has to be pointed out that, due to our experimental conditions (using saturating substrate concentrations and an incubation mixture optimal for each of the tested enzymes) we measured maximal ATPase activities in our tests. Our data, therefore, provide information on changes in the size of the ATPase fraction (among the membrane proteins) rather than the *in vivo* ATPase activity. We, therefore, can conclude that all our experi-

mental plants build membrane transport capacity as a response to salt stress. Surplus transport capacity can be reduced as soon as ion homeostasis of the cytosol is achieved. This reduction of ATPase activity was observed earlier in halophytes than in glycophytes. – Similar effects of salt treatments on ATPase activity have been observed in plant roots by Koyro *et al.* (1993).

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