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# Dynamics of tonoplast proton pumps and other tonoplast proteins of *Mesembryanthemum crystallinum* L. during the induction of Crassulacean acid metabolism

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**Abstract.** In plants of *Mesembryanthemum crystallinum* the activities of the two proton pumps on the tonoplast, i.e. the ATPase and the pyrophosphatase, and the gel-electrophoretic pattern of the total tonoplast proteins were analyzed during the transition of the metabolic state from $C_3$ photosynthesis to Crassulacean acid metabolism (CAM). In one series, CAM was induced by watering the plants with NaCl. In another series, the change of the metabolic state to CAM was a consequence of the aging of the plants. No significant differences in the specific activities of ATP hydrolysis were found in plants performing $C_3$ photosynthesis and CAM, respectively. However, with both series the protein content of tonoplast preparations and, in parallel, the total ATP hydrolytic activity of the tonoplast ATPase were higher after the change to CAM. In contrast, the specific activity of pyrophosphate hydrolysis was maximum in the preparations of young plants and diminished after the induction of CAM in both series. Therefore the tonoplast ATPase seems to be the main enzyme responsible for the energization of malate accumulation in CAM. The tonoplast pyrophosphatase is important in the early stages of plant growth and plays a minor role in CAM. With *M. crystallinum* the change from $C_3$ photosynthesis to CAM is accompanied by de-novo synthesis of tonoplast proteins. Several polypeptides with relative molecular masses ($M_r$s) of 55, 41, and 36 kDa were clearly more pronounced in the gel-electrophoretic pattern of the total tonoplast protein after CAM induction. These changes were independent of the CAM-inducing salt treatment or aging. Moreover, two subunits of the tonoplast ATPase with $M_r$s of about 27 and 31 kDa showed particularly high intensities only in the CAM state. It is assumed that the subunit composition of the tonoplast ATPase differs in the two metabolic states and that the two subunits induced modify the regulation of the ATPase in CAM. In addition, the reaction of the plants to the NaCl treatment per se was the induction at the tonoplast of a polypeptide with an $M_r$ of 24 kDa.

**Key words:** ATPase – Crassulacean acid metabolism – *Mesembryanthemum* – Polypeptide induction – Pyrophosphatase – Salt stress – Tonoplast

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*Abbreviations:* CAM = Crassulacean acid metabolism; $M_r$ = relative molecular mass

## Introduction

Induction of Crassulacean acid metabolism (CAM) in *Mesembryanthemum crystallinum* is accompanied by drastic changes in the day-night flux of metabolites within the cells and especially by changes in the transportation of solutes across the tonoplast. For this reason *M. crystallinum* provides an ideal plant material for the investigation of the function and regulation of transport proteins at the tonoplast. One of the key processes of CAM during the night phase is the accumulation of malic acid in the vacuole. It is well established that the storage of malic acid is driven by an electrochemical proton gradient (Lüttge and Ball 1979; Lüttge et al. 1981) which is built up by a vacuolar ATPase (Aoki and Nishida 1984; Jochem et al. 1984). Investigations of the subunit structure of several tonoplast vacuolar-type (V-type) ATPases always revealed two subunits of relative molecular masses ($M_r$s) about 68 and 58 kDa (summarized by Forgac 1989), which were shown to constitute a cytoplasmic domain of the enzyme with the catalytic center of ATP hydrolysis (Rea et al. 1987) and a possible regulatory function, respectively (Mandala and Taiz 1986). An additional subunit of about 16 kDa is believed to form the proton channel through the membrane (Moriyama and Nelson 1989). The number and the molecular mass of further subunits described are quite different for the V-type ATPases from several plant and fungal sources (Forgac 1989). The V-type ATPase of green leaf cells of plants performing CAM, i.e. *Kalanchoë daigremontiana* and *M. crystallinum* was shown to consist of at least six

subunits (Bremberger et al. 1988). Initially these results seemed to be at variance with the composition of the tonoplast ATPase from other plant sources which consisted mostly of three subunits (summarized by Mandala and Taiz 1986; Forgac 1989). However, more recently other authors have also found up to nine subunits for the V-type ATPase of non-green plant tissues, as is the case for the ATPase from animal sources: in addition to the three subunits already described one subunit of about 100 kDa and up to five subunits in the range of 19–52 kDa are reported (Parry et al. 1989 and references therein). The function of these subunits is unknown.

Besides the ATPase, an electrogenic proton-translocating pyrophosphatase was identified and isolated from the tonoplast of higher plants (Maslowski and Maslowska 1987; Sarafin and Poole 1989). The physiological importance of this second proton pump is not clear. Several functions have been suggested: energetization of membrane transport, regulation of the intracellular concentration of pyrophosphate, and salvage of the free energy generated by pyrophosphohydrolytic reactions of polymer synthesis (Rea and Sanders 1987). In the CAM plant *K. daigremontiana,* the pyrophosphatase was shown to regulate the activity of the tonoplast ATPase (Marquardt and Lüttge 1990).

The present communication shows the results of an investigation of the dynamics of the tonoplast proteins during the induction of CAM in *M. crystallinum.* It evaluates the influence of the salt stress used for CAM induction on these proteins and their enzymatic activity. For this purpose, the activity of the two proton pumps at the tonoplast and the gel-electrophoretic pattern of the total tonoplast protein – with special regard to the subunit structure of the tonoplast ATPase – were correlated with the salt treatment of the plants and with the time-course of CAM induction, respectively.

## Material and methods

*Plants.* Seedlings of *M. crystallinum* L. were grown from seeds of our own collection in soil culture as described by Struve et al. (1985). During the light phase of 14 h, the plants were illuminated with 350–500 μmol photons · $m^{-2}$ · $s^{-1}$, the temperature was 25° C and the relative humidity 50–60%. During the dark phase of 10 h, the temperature was 15° C and the relative humidity was 70–80%. Crassulacean acid metabolism was induced by NaCl stress (Winter and Lüttge 1979). After the appearance of the third leaf pair (about six weeks after sowing) the plants were watered with NaCl solutions. The NaCl concentration was increased every second day in steps of 100 mM until a concentration of 400 mM was reached, and was then kept at this level. All plants used for the series of experiments described in this paper derived from the same batch of seeds.

*Cell sap and solutes.* Cell sap of mesophyll cells was prepared as described by Smith and Lüttge (1985). The malate concentration in the sap was measured enzymatically according to Hohorst (1970). The concentration of sodium was determined by flame-photometry (Netheler und Hinz, Hamburg, FRG).

*Preparation of tonoplast vesicles.* Leaves were harvested at the end of the light phase, and protoplasts were isolated by enzymatic digestion of the leaf tissue with 0.5% (w/v) Cellulase "Onozuka" RS (Yakult Honsha Co., Tokyo, Japan) and 0.02% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical Co., Tokyo, Japan) as described by Struve and Lüttge (1987). Tonoplast vesicles were prepared after homogenization of the protoplasts by density-gradient centrifugation on a 24% sucrose cushion according to Klink et al. (1989). These methods were modified by adjusting the osmotic pressure of all solutions for the treatment of intact protoplasts with mannitol to the osmotic pressure in the cell sap of the corresponding samples. Thus, changes in the yield of tonoplast membranes resulting from lysis of the protoplasts by osmotic rupture were avoided.

*Protein assay.* Protein was assayed using Amido Black 10 according to the procedure described by Popov et al. (1975).

*Assay of the hydrolysis of ATP and pyrophosphate.* Pyrophosphate and ATP were hydrolysed in reaction mixtures as described by Marquardt and Lüttge (1987) and Struve et al. (1985), respectively, in a final reaction volume of 0.5 ml and in the presence of 0.01% (w/w) Triton X-100 to reveal latent activity. Anions were added in order to characterize the preparations (Sze 1985; Al-Awquati 1986): at pH 8.0, 50 mM chloride (potassium salt) stimulates the vacuolar ATPase; 50 mM nitrate (sodium salt) partially inhibits the vacuolar and mitochondrial ATPases. As the mitochondrial ATPase is completely inhibited by 1 mM azide (sodium salt), the activity of the tonoplast ATPase was estimated as the nitrate-sensitive, azide-resistant part of ATP hydrolysis. At pH 6.5, the plasmalemma ATPase is completely inhibited by 0.1 mM vanadate (sodium salt). The phosphate released by the enzymatic reactions was determined according to Lin and Morales (1977).

*Purification of the tonoplast ATPase.* Tonoplast proteins were solubilized using 2% (v/v) of the detergent Triton X-100. The tonoplast ATPase was purified using size-exclusion and ion-exchange chromatography as described by Bremberger et al. (1988).

*Electrophoresis.* Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out using the method of Laemmli (1970) in 16 cm × 16 cm slab gels of 10% (w/w) acrylamide (5% (w/w) N, N'-methylene-bis-acrylamide). After the run, the gels were stained for proteins with silver according to the method of Oakley et al. (1980). The $M_r$s of polypeptides were calculated from several gels using molecular-mass standards from Sigma, Munich, FRG.

## Results and discussion

*Characterization of plant metabolism.* The metabolic status of the *Mesembryanthemum* plants used for the preparation of tonoplast vesicles was investigated during the time course of an experiment by measuring the concentrations of sodium in the cell sap (Fig. 1a) and the day-night differences in the concentrations of malate (Fig. 1b). The sampling intervals were especially chosen so that the time of CAM induction of the salt-treated plants was covered. As has already been described by Winter and von Willert (1972), the salt treatment resulted in an accumulation of sodium in the cell sap to a concentration of more than 800 mM, while the level in the non-treated plants remained constantly low (Fig. 1a). In response, the salt-treated plants began to accumulate malate during the dark phase some days after the beginning of the treatment (Fig. 1b), a typical indication of the occurrence of CAM. The non-treated control plants from the same batch of seedlings also exhibited a nocturnal accumulation of malate as a consequence of aging (Winter 1973) but the switch to CAM started about 10 d later than in the salt-treated plants and the maximum

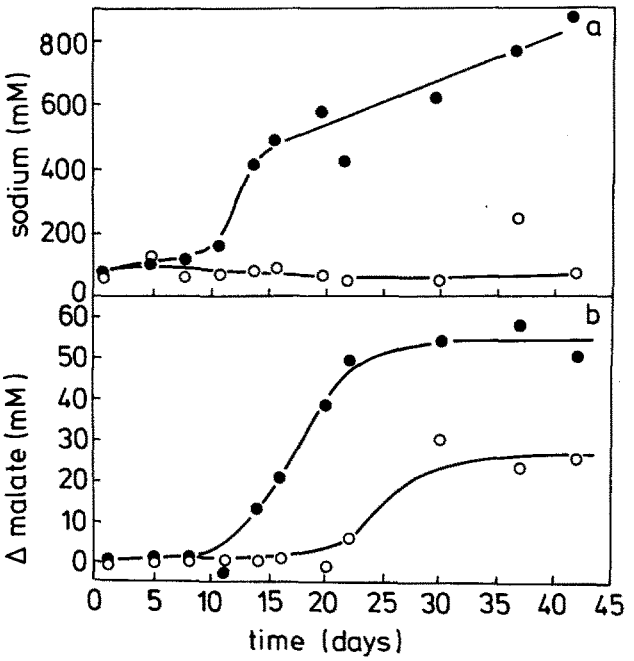


**Fig. 1a, b.** Concentration of sodium (**a**) and △ malate (**b**; concentration in the samples at the end of the dark phase minus concentration at the end of the following light phase) in the cell sap of *Mesembryanthemum* plants either treated *(closed symbols)* or not treated *(open symbols)* with NaCl. Day 0 indicates the beginning of the salt treatment

day-night differences in malate concentration were smaller. These two series of plants provided preparations of tonoplast membranes which allowed us to differentiate between changes of tonoplast proteins caused by the switch from $C_3$ photosynthesis to CAM and changes induced by the salt treatment itself.

*Characterization of the tonoplast preparations.* Table 1 shows the contributions of ATP hydrolysis derived from different cellular membranes as measured by the effects of ATPase inhibitors (Sze 1985; Al-Awquati 1986): in the preparations of both, NaCl-treated and non-treated plants, ATP hydrolysis at pH 8.0 was highly stimulated by chloride; in both cases, the contribution of the nitrate-sensitive, azide-resistant ATP hydrolysis was higher than 60% (Table 1). Stimulation by chloride and inhibition by nitrate are both characteristics of the vacuolar ATPase. Taking into account that the vacuolar ATPase is not completely inhibited by 50 mM nitrate, e.g. a reduction of the activity of 50% was found in *Neurospora crassa* (Bowman and Bowman 1986) and a reduction of 55% (tonoplast vesicles) and 88% (purified ATPase) in *Beta vulgaris* (Parry et al. 1989), our results indicate a high contribution of the tonoplast ATPase to the total ATPase activity and a high enrichment of tonoplast in the preparations. This remained constant in all preparations. The contamination with azide-sensitive ATP hydrolysis (mitochondrial ATPase) was low and also constant in the tonoplast preparations of both plant series. In contrast, the contribution of vanadate-sensitive ATP hydrolysis measured at pH 6.5 (plasmalemma ATPase)

**Table 1.** Effects of anions and inhibitors on ATP hydrolysis by tonoplast preparations from *Mesembryanthemum* plants treated (+NaCl) or not treated (−NaCl) with salt: contribution of chloride-stimulated (controls without chloride), nitrate-sensitive and azide-resistant (tonoplast ATPase), and azide-sensitive (mitochondrial ATPase) ATP hydrolysis as % of total ATP hydrolysis measured at pH 8.0. These values are means ± SD of the measurements of ten preparations from days 1 to 37 (three independent measurements of each preparation). The contribution of vanadate-sensitive ATP hydrolysis (plasmalemma ATPase) was measured at pH 6.5, and the values are means ± SD for preparations of older plants exhibiting a nocturnal accumulation of malate both in NaCl-treated (days 11–37; six preparations) and non-treated plants (days 22–42; three preparations)

| | ATP hydrolysis as % of total | |
|---|---|---|
| | +NaCl | −NaCl |
| Chloride-stimulated | 70 ± 20 | 75 ± 12 |
| Nitrate-sensitive (azide-resistant) | 61 ± 8 | 65 ± 11 |
| Azide-sensitive | 14 ± 9 | 6 ± 5 |
| Vanadate-sensitive | 13 ± 5 | 21 ± 7 |

was more than 40% in preparations of young plants (days 0 to 8) where the vacuoles apparently were not fully developed. It decreased as the plants aged and remained at a constant low level after the beginning of malate accumulation in both salt-treated and control plants (Table 1). This change could be caused either by the relatively low contribution of tonoplast to the total amount of different membranes in the young plants or by a shift of the specific mass of the plasmalemma during growth of the leaves. If the specific mass of the plasmalemma was initially similar to that of the tonoplast, then the plasmalemma might not have been well separated from tonoplast vesicles during the gradient centrifugation. The decrease in the contribution of vanadate-sensitive ATP hydrolysis during the time course was considerably lower in the non-treated control than in salt-treated plants, probably due to differences in the composition of the tonoplast preparations in the two series. Another possible explanation of this phenomenon might be the occurrence of vanadate-sensitive ATP hydrolysis at the tonoplast during certain stages of the growth *M. crystallinum* plants as shown for *Acer pseudoplatanus* (Montrichard et al. 1989).

*Activities of ATPase and pyrophosphatase.* The hydrolytic activity of the tonoplast ATPase (nitrate-sensitive and azide-resistant) measured in the preparations of the salt-treated plants was more than sixfold higher in the preparations from days 11 to 22 than in the preparations from days 0 to 8 before a nocturnal accumulation of malate was found (Table 2). Of the plants grown without salt treatment, the higher activity was also found in those plants exhibiting some degree of CAM (days 22 to 42). This indicates that the increase in the absolute quantity of ATP hydrolysis of the tonoplast ATPase in the preparations was correlated with the induction of CAM and was independent of the CAM-inducing NaCl or aging.

**Table 2.** Total activities of the nitrate-sensitive, azide-resistant ATP hydrolysis measured at pH 8.0, and protein concentrations in the tonoplast preparations from *Mesembryanthemum* plants treated (+NaCl) or not treated (−NaCl) with salt. The values are related to volume units and are comparable since all preparations, of the same initial leaf fresh weight were resuspended in the same volume of buffer. Data are means ± SD of three preparations from day 0 until day 8, when the salt-treated plants performed $C_3$ photosynthesis, and of five preparations from day 11 to day 22, when the salt-treated plants performed CAM. Data were analysed by the student's t-test and means followed by the same letters are not statistically significantly different from each other; means followed by different letters are statistically different at the 0.1% level

| Days | ATP hydrolysis ($\mu mol \cdot h^{-1} \cdot ml^{-1}$) | | Protein concentration ($\mu g \cdot ml^{-1}$) | |
|---|---|---|---|---|
| | +NaCl | −NaCl | +NaCl | −NaCl |
| 0–8 | 1.6 ± 1.0a | 1.6 ± 0.6a | 85 ± 44c | 78 ± 33c |
| 11–22 | 10.6 ± 1.7b | 4.7 ± 2.5a | 327 ± 33d | 137 ± 64c |

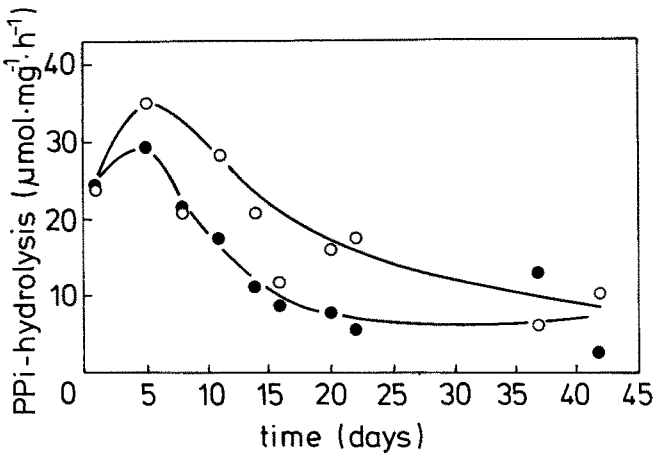


**Fig. 2.** Specific activity of pyrophosphate ($PP_i$) hydrolysis in tonoplast preparations from NaCl-treated *(closed symbols)* and non-treated *(open symbols) Mesembryanthemum* plants. Day 0 indicates the beginning of the salt treatment

With the induction of CAM, protein concentrations in the tonoplast preparations increased together with the ATP hydrolysis (Table 2). This indicates the de-novo synthesis of tonoplast proteins during the induction of CAM. Since protein concentration and ATP hydrolysis both increased, differences in the specific activity of ATP hydrolysis between the salt-treated plants and the controls were not significant during corresponding periods.

For both NaCl-treated and non-treated plants the specific activity of pyrophosphate hydrolysis (Fig. 2) was maximal at the beginning of the time course and decreased with the plant age. In addition, the activity of the pyrophosphatase in the tonoplast preparations from salt-treated plants was always somewhat lower than in the non-treated plants during the time at which the metabolism (days 11 to 20) changed from $C_3$ photosynthesis to CAM. This corresponds to earlier results (Bremberger et al. 1988; Marquardt-Jarczyk and Lüttge 1990) showing that there was little or no activity of the tonoplast pyrophosphatase when *M. crystallinum* was in the CAM state.

*Gel-electrophoresis of tonoplast proteins.* Gel-electrophoresis of the tonoplast preparations (Fig. 3) showed many changes in the polypeptide pattern during the time course of NaCl treatment. Over a wide range of $M_r$s there were numerous polypeptides appearing or disappearing, some of which were present only during some stages of plant growth. It is noteworthy that these changes occurred gradually in sequential preparations. None of the polypeptides was correlated with the level of contamination of the preparations as evaluated by inhibitors of ATP hydrolysis, since this contamination occurred at random during the time course. For both treated and non-treated plants there were many weakly stained polypeptides present in the early samples. The most prominent changes became detectable after day 11 for salt-treated plants (Fig. 3a) and on day 20 for the controls (Fig. 3b), i.e. just before a nocturnal accumulation of malate was measured in the corresponding samples (Fig. 1a). During this stage of plant growth, the number of minor, diffusely stained polypeptides decreased, whereas some distinct polypeptides became even more clearly pronounced. As the same amount of protein was used for electrophoresis of all tonoplast preparations, these results indicate that the amounts of some polypeptides increased markedly relative to the total tonoplast protein. These polypeptides were induced in the tonoplast preparations of both series of plants, independent of the CAM-inducing salt treatment or aging of the plants.

In order to identify the subunits of the tonoplast ATPase, samples of the highly purified enzyme from plants of *M. crystallinum* in the CAM state (Bremberger et al. 1988) were run on the same gels, where the specific activity of ATP hydrolysis was 230 $\mu mol \cdot mg^{-1} \cdot h^{-1}$, was insensitive to azide and to vanadate, and inhibited to 95% by nitrate. Most subunits of the purified ATPase appeared in all tonoplast preparations over the whole time course. In contrast two subunits with $M_r$s of 31 and 27 kDa became much more abundant in the tonoplasts of both NaCl treated and control plants at the beginning of the metabolic switch from $C_3$ photosynthesis to CAM, i.e. on day 11 (Fig. 3a) and day 20 (Fig. 3b), respectively. The subunit of 27 kDa corresponds to the subunit of 24 kDa formerly described in Bremberger et al. (1988). Since the levels of these subunits increased relative to other subunits of the purified ATPase of plants in the CAM state, it follows that the tonoplast ATPase exhibited two additional subunits in plants in the CAM state.

In both series of plants other polypeptides of $M_r$s 55, 41 and 36 kDa appeared in the protein pattern of the tonoplast preparations on days 11 (Fig. 3a) and 20 (Fig. 3b), respectively. The fact, that these polypeptides became visible in relation to CAM-induction but did not belong to the $H^+$-transporting ATPase indicates that they probably function in other transport processes across the tonoplast that are necessary for CAM.

Comparison of the gel-electrophoretic pattern of the tonoplast proteins from the plants treated with salt with that of the controls showed that there was one polypeptide of about 24 kDa which appeared only in the salt-treated plants. The induction of this polypeptide was therefore a reaction of the plants to the NaCl treatment.

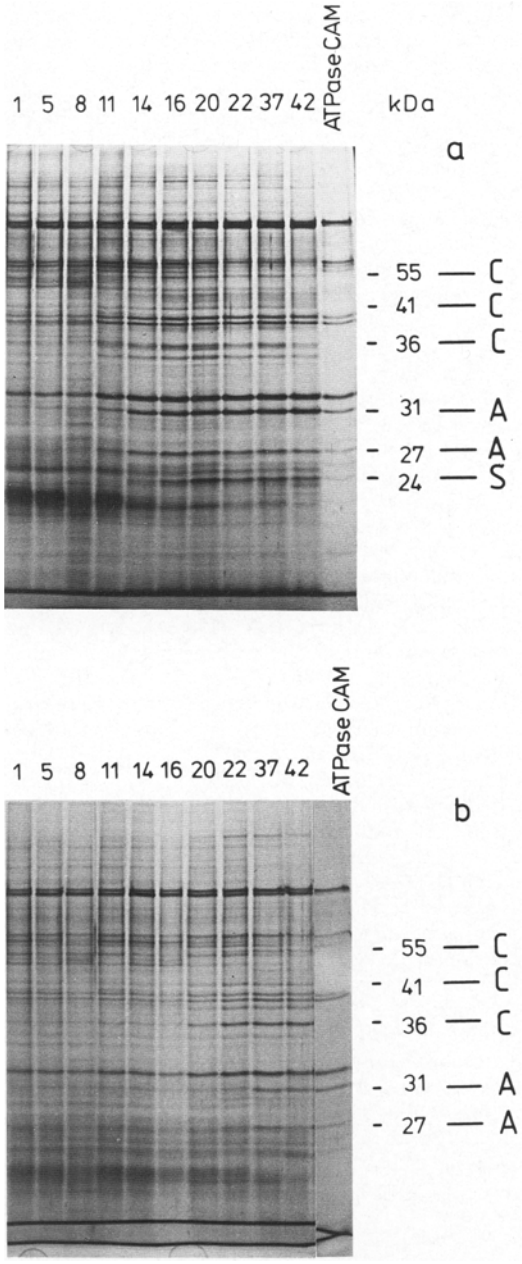


**Fig. 3a, b.** Gel-electrophoretic pattern (10% acrylamide w/w) of the tonoplast proteins (1 µg protein each gel) from NaCl-treated (**a**) and non-treated (**b**) *Mesembryanthemum plants*. The numbers at the top of the gels indicate the day after the beginning of salt treatment on which the samples were prepared. *ATPase CAM:* purified tonoplast ATPase from plants in the CAM state (0.3 µg protein). Numbers on the right-hand side of the figure indicate the $M_r$s of polypeptides induced during the experiment. *A:* ATPase-related polypeptides induced during the switch to CAM; *C:* non-ATPase-related peptides induced during the switch to CAM; *S:* polypeptide induced by salt treatment. The gels were stained for proteins with silver according to Oakley (1980)

The induction of polypeptides with a similar molecular mass during the adaption to salt stress has been found for several other species but, until now, this peptide has been correlated with the tonoplast membrane only in *Hordeum vulgare* (Hurkman et al. 1988). The function of these polypeptides is unknown.

## Conclusions

In *M. crystallinum* the switch to CAM is not achieved by simple kinetic regulation of transport proteins existing in the state of $C_3$ photosynthesis. The induction of CAM is accompanied by the de-novo synthesis of tonoplast proteins, and the relative amounts of some distinct polypeptides, including subunits of the tonoplast ATPase, become markedly pronounced. Most probably the newly synthesized proton pump is another form of the ATPase which already exists in the $C_3$ state, but with two additional subunits. The protein composition of the tonoplast changes together with the transition of the metabolic pathway, and any influence of the salt-treatment or of the plant age on the differences brought about by the induction of CAM can be excluded by the comparison with the control series.

An earlier characterization of the activity of the tonoplast ATPase of *M. crystallinum* showed different enzyme kinetics in the two metabolic states: the activity of ATP hydrolysis was increased during the transition from $C_3$ photosynthesis to CAM; the hydrolytic activity of the enzyme from plants in the CAM state was stimulated by malate in the presence and in the absence of Triton X–100, whereas the enzyme of plants in the $C_3$ state was stimulated by malate only in the presence of Triton X–100. The pH optimum of the ATPase of plants in the CAM state was more basic than that of the ATPase from plants in the $C_3$ state (Struve et al. 1985; Struve and Lüttge 1987), but it was the same after solubilization of the ATPase (compare Fig. 7 in Struve and Lüttge 1987 with Fig. 3 in Struve and Lüttge 1988). The fact that these kinetic differences are influenced by the presence of detergent indicates that the two ATPase subunits induced during the transition to CAM are probably involved in the interactions between the enzyme and the membrane and in the regulation of ATPase activity. This also relates to the observations of Klink et al. (1989) who found that the area covered by the tonoplast ATPase in freeze-fractures suspensions of tonoplast vesicles from leaves of *M. crystallinum* was higher in the CAM state than in the $C_3$ state. If the subunits induced are indeed involved in the interactions of the enzyme with the membrane and in the regulation of the ATPase, the different subunit of the ATPase in the two metabolic states – like the different subunit composition reported for the vacuolar ATPase from other sources – probably reflects the changed acidification requirement in the vacuole.

The CAM-inducing salt treatment of the plants neither affected the activity of the proton pumps at the tonoplast nor the concentration of protein in the tonoplast fractions. Nevertheless, at the level of tonoplast proteins, the adaptation of the plants to salt stress appeared to be linked to the induction of a new polypeptide of $M_r$ about 24 kDa.

The tonoplast pyrophosphatase of *M. crystallinum* was highly active during the period of high growth rates of the plants. Its activity diminished after the induction of CAM and therefore it seems not to be involved in transport processes specific for CAM or in a regulation of the tonoplast ATPase as shown for the CAM plant *K. daigremontiana:* the basic rates of ATP-dependent

proton transport in *M. crystallinum* were higher and already in the range of the pyrophosphatase-stimulated rates obtained with *K. daigremontiana* (Marquardt-Jarczyk and Lüttge 1990). In contrast to *K. daigremontiana*, therefore, it is unlikely that in *M. crystallinum* in the adult state the pyrophosphatase functions in the energetization of malate transport or the regulation of the tonoplast ATPase. The results indicate that the pyrophosphatase is highly active in young and still-growing plants where the concentration of pyrophosphate derived from polymer synthesis can be assumed to be high. This would agree with the findings and conclusions of Chanson and Pilet (1987) for the tonoplast pyrophosphatase of maize roots, where the activity decreases with the differentiation of the tissue.

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