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Short sequence-paper

cDNA sequence and expression of subunit E of the vacuolar H^+-ATPase in the inducible Crassulacean acid metabolism plant *Mesembryanthemum crystallinum* [1]

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Abstract

A cDNA coding for subunit E of the vacuolar H^+-ATPase was cloned from *Mesembryanthemum crystallinum*, a plant which switches from C3-photosynthesis to Crassulacean acid metabolism under saline growth conditions. Sequence homology between the three subunit E-polypeptides of different higher plant species varied between 77.6 and 73.3%; peptide length was between 226 and 230 amino acid residues, 43 of which are invariant in all seven subunit E-polypeptides known so far from animals, fungi and plants. The deduced relative molecular mass of subunit E in *Mesembryanthemum crystallinum* is 26 162 Da. Subunit E is present both in C3- and CAM-plants. mRNA levels increased severalfold in leaves of CAM-induced plants. This was accompanied by a less pronounced increase in subunit E protein. Obviously, expression is stimulated under conditions of increased requirement for tonoplast H^+-pumping activity.

Keywords: Crassulacean acid metabolism; Expression (tissue, stress); ATPase, H^+-; Salt stress; Subunit E; Tonoplast; Vacuole; (*M. crystallinum*)

The vacuolar-type H^+-ATPase occurs in all eucaryotic cells and excretes protons from the cytoplasm into the vacuoles and lysosomes. As a consequence of the action of the H^+-ATPase, the vacuolar lumen acidifies, and an inside positive membrane potential is established. The acidic pH of the vacuolar lumen, the membrane potential across the vacuolar membrane and the resulting proton-motive force are important parameters to realize the specific physiological functions of the vacuoles. Large efforts have been made to biochemically characterize the vacuolar H^+-ATPase of higher plants [1–4]. However, the complex molecular structure of the higher plant H^+-ATPase is only beginning to be elucidated on the level of the proteins and the genes. Information is available for subunits A, B and c. Recently we have reported the first sequence for a subunit E-homologue of a higher plant, the monocot *Hordeum vulgare*, and demonstrated that subunit E-protein is part of the structure of the vacuolar H^+-ATPase [5]. However, the distribution among species and expression of subunit E has not been studied in detail.

The limited knowledge concerning the structure of the plant ATPase contrasts the molecular information available for the yeast H^+-ATPase. In yeast, the V-type ATPase is composed of at least 11 subunits [6]. A requirement for expression of ATPase activity and assembly of the complex has been demonstrated for six other polypeptides [7]. A similarly complex structure and assembly process may be assumed in higher plants. A number of research groups have purified H^+-ATPase protein from various plant sources, for instance barley, mung bean and oat [8–11]. After separation of these homogeneous plant H^+-ATPase proteins by denaturing SDS-PAGE, 8 to 10 subunits of distinct molecular masses between 110 and 9 kDa were identified.

Until now, sequence information on subunit E was available for V-type ATPases from two vertebrates, one insect, and from yeast [12–15]. Therefore we extended our investigation from the monocot barley [5] to the dicot

Abbreviations: A.t., *Arabidopsis thaliana*; CAM, Crassulacean acid metabolism; H.v., *Hordeum vulgare*; M.c., *Mesembryanthemum crystallinum*; TpP31, 31 kDa tonoplast protein.

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[1] The subunit E-cDNA sequences have been submitted to the EMBL Data Library under the accession numbers E. X92117 and E. X92118.

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Mesembryanthemum crystallinum, an established model system for studying the molecular biology of plant adaptation to salt stress.

A cDNA library constructed from *Mesembryanthemum crystallinum* in the CAM state was screened with a cDNA fragment of TpP31Hv [5]. Nucleotide and derived amino acid sequences of the longest cDNA clone are depicted in Fig. 1. The open reading frame extends over 678 bp of the 1070 bp. We also cloned a subunit E-cDNA of *Arabidopsis thaliana* (TpP31At; data not shown). On the level of the amino acid sequence, identity was 77.6% and 73.6% between TpP31Hv and TpP31Mc, and TpP31Hv and TpP31At, respectively. The cDNA-derived amino acid sequences of TpP31Hv, At and Mc are aligned with subunit E of the vacuolar ATPase of man in Fig. 2. Eighty amino acids are strictly conserved between plants and man. The same conservation is observed between the plant proteins and subunit E of *Manduca sexta* and *Bos bovis*. Conversely, homology is least with the subunit E of yeast (cf. Ref. [5]). By now, sequence information for subunit E is available for a total of 7 species. 43 amino acids are invariant. The deduced molecular mass of subunit E of *M.*

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   1  ACG AGG TTG AAC CAA AGC AGC AGC AGC AAA TCC ACT CTA GAG AGA GAA    48

   1                                      M   N   D   T   D   V   Q     7
  49  AAA TTC AAA AAC TGA CCC GAT CCC AAA ATG AAC GAC ACC GAT GTC CAA    96

   8  N   Q   I   Q   Q   M   V   R   F   M   R   Q   E   A   E   E    23
  97  AAC CAG ATC CAG CAG ATG GTC AGA TTC ATG CGC CAA GAA GCG GAG GAG   144

  24  K   A   N   E   I   S   V   S   A   E   E   E   F   N   I   E    39
 145  AAG GCC AAC GAG ATC TCT GTC TCT GCC GAA GAA GAA TTC AAC ATT GAG   192

  40  K   L   Q   L   V   E   A   E   K   K   K   I   R   Q   E   Y    55
  93  AAG TTG CAA TTA GTT GAA GCG GAG AAG AAG AAG ATC AGG CAA GAG TAT   240

  56  E   R   K   A   K   Q   V   D   V   R   R   K   I   E   Y   S    71
 241  GAG CGC AAG GCG AAG CAA GTG GAC GTT CGG AGG AAA ATT GAG TAC TCT   288

  72  M   Q   L   N   A   S   R   I   K   V   L   Q   A   Q   D   D    87
 289  ATG CAG CTC AAT GCT TCT CGG ATC AAG GTT CTT CAA GCT CAG GAT GAT   336

  88  L   V   N   A   M   K   E   A   A   S   K   E   L   L   L   V   103
 337  TTA GTC AAT GCA ATG AAA GAG GCT GCA TCC AAA GAG CTG CTG CTT GTA   384

 104  S   G   D   H   H   Q   Y   R   N   L   L   K   E   L   I   V   119
 385  AGT GGT GAC CAT CAC CAA TAC AGG AAC CTC CTG AAA GAG CTC ATT GTT   432

 120  Q   S   L   L   R   L   K   E   P   A   V   L   L   R   C   R   135
 433  CAG AGT TTG CTT AGA CTG AAA GAA CCA GCT GTC TTG TTG CGT TGC CGA   480

 136  E   E   D   K   H   H   V   H   R   V   L   H   S   A   R   E   151
 481  GAA GAG GAT AAG CAC CAC GTG CAC CGT GTC CTC CAT TCT GCT AGG GAA   528

 152  E   Y   G   E   K   A   C   V   S   H   P   E   V   I   V   D   167
 529  GAG TAT GGA GAA AAA GCT TGT GTT AGT CAT CCT GAG GTC ATA GTT GAC   576

 168  D   I   H   L   P   P   A   P   T   S   Y   D   S   H   E   L   183
 577  GAC ATC CAT CTT CCA CCT GCT CCT ACT AGT TAT GAT TCT CAT GAA CTT   624

 184  S   C   S   G   G   V   V   M   A   S   R   D   G   K   I   V   199
 625  TCT TGC TCT GGC GGT GTT GTT ATG GCT TCT AGA GAT GGC AAG ATT GTG   672

 200  F   E   N   T   L   D   A   R   L   E   V   A   F   R   K   K   215
 673  TTT GAG AAC ACT CTT GAT GCT AGA CTG GAG GTT GCA TTC AGG AAG AAG   720

 216  L   P   Q   I   R   K   Q   L   F   A   V                       226
 721  CTC CCT CAG ATC CGC AAG CAG CTC TTT GCT GTA TGA TGA GTT GTG ATC   768

 769  AGA GGA GTG TCA TTT GTT CCG GGT TAC TGA GGC TGC TTT GAT ATG GGG   816

 817  TAT GGT CAT TGA CCA CAG TGA GCT GTT TGG TCA CCT GTC TTT GAG TCC   864

 865  ACT ACC AAT CTT ATT TAT CTG TTT GCG TGC CAA TAT GTT GAT TAG TAA   912

 913  GTA CAT GAA CAA TTT TCT GCA TTC TTG ATT ATA TTA AAT GAG GTG TAT   960

 961  TCA GAA TAC TCA AAT TTT TTT CCC AGT TTA TGG TCA ATC AAT AAA ATA  1008

1009  AAT GGC AGC TTC TGA TGA AAC GCT GCA TTT TAT GTA AAA AAA AAA AAA  1056

1057  AAA AAA AAA AAA AA                                              1070
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Fig. 1. Nucleotide sequence and derived amino acid sequence of the *Mesembryanthemum crystallinum* TpP31-cDNA. A cDNA unizap-library was synthesized from *M. crystallinum* leaf mRNA [26,27]. 250000 pfu were screened by hybridization with TpP31Hv. 34 positive clones were obtained; 15 were selected for in vivo excission. Sequence analysis was performed with the largest cDNA-insert of 1070 basepairs.

crystallinum is 26 126 Da which is in the size range of all reported subunit E polypeptides.

Mesembryanthemum crystallinum is a salt-tolerant plant which switches from C3-photosynthesis to Crassulacean acid metabolism under salt and drought stress [16]. Concomitantly, the H^+-pump density and capacity increase at the tonoplast [17]. Induction of CAM was monitored by the increase in PEP carboxylase protein. 5-week-old plants were irrigated with solutions of increasing NaCl concentrations. Young and old leaves were analyzed by Western blotting for protein amounts of subunit E and A, and PEP carboxylase 8 d after beginning of the salt treatment (Fig. 3). During this period, the leaf metabolism switches from C3-photosynthesis to CAM depending on leaf age and salt concentration [18]. PEP carboxylase protein was on a very low level in young leaves of plants grown at 0 or 100 mM NaCl. It was little increased at 200 mM NaCl, and moderately induced at 400 mM NaCl. In old leaves (second pair emerged after the cotyledons), PEP carboxylase was also low in the presence of 0 and 100 mM NaCl but strongly induced in 200 and 400 mM NaCl. Salt-dependent changes in amount of subunit E protein were much less pronounced. Nevertheless, an increase in subunit E protein was reliably observed with increasing salt concentration. Similar to PEP carboxylase, subunit E levels were higher in old than in young leaves suggesting a relation with CAM expression.

Transcript levels for subunit E as visualized in Northern type of hybridization increased both in young and old leaves of *M. crystallinum* plants adapted to high salt concentrations. Transcript levels were low in control plants grown without NaCl. The hybridization signal increased in dependence of the NaCl concentration. The transcript levels increased by a factor of about 5 between plants grown at 0 and 400 mM NaCl (Fig. 4). In old leaves, the transcript accumulated about 8-fold when comparing plants stressed with 400 mM NaCl and controls without salt. There was only a slight difference in subunit E mRNA-levels between plants grown in 200 or 400 mM NaCl. This contrasts the RNA-level of PEP carboxylase which revealed the strongest increase between 200 and 400 mM NaCl in the irrigation water.

As pointed out above, detailed structural analysis in a variety of species has demonstrated that the subunit composition of the V-type ATPase is complex [2], possibly similar to yeast [7]. Information on the primary structure of subunit E is now available for seven species, i.e. *Homo sapiens, Bos bovis, Manduca sexta, Saccharomyces cerevisiae, Hordeum vulgare, Mesembryanthemum crystallinum* and *Arabidopsis thaliana* (own results, unpublished data). Several independent results show that subunit E is a constitutive part of the V-type H^+-ATPase. (i) Genetic information is detected in all species analysed so far. (ii) On the protein level, polypeptides immunologically

Fig. 2. Sequence alignment of subunit E polypeptides of the vacuolar H^+-ATPase. Alignment of amino acid sequences of subunit E of *Arabidopsis thaliana* (accession number, X92117), *Hordeum vulgare* [5], *Mesembryanthemum crystallinum* and *Homo sapiens* [14]. Positions of asterisks indicate conserved amino acid residues.

crossreactive to TpP31Hv are present in all tested plant species ([19] and Dietz, K.-J., unpublished data). (iii) In C3 plants such as rape, subunit E is immunologically detected in all tissues, and its protein amount is fairly constant on a fresh weight basis in all living tissues. (iv) A polypeptide of about 32 kDa has been described in many preparations of V-type H^+-ATPases [8–11,17,21].

The transition of *Mesembryanthemum crystallinum* from the C3 mode of photosynthesis to CAM is characterized by a specific temporal pattern of biochemical and molecular changes. Upon initiating the transition by irrigation with salt solutions, the fastest response is seen in the accumulation of proline. Maximum proline levels are reached after two days. At this time, the activity of PEP carboxylase starts to increase. The increase in activity is regulated mainly on the transcriptional level. *ppc* mRNA levels are low in non-salt-treated control plants and are maximal three days after beginning the salt treatment [20]. The transition to CAM also involves a 2- to 3-fold increase in V-type ATPase activity [17] which is also caused by an increase in protein amount. SDS PAGE analysis of purified ATPase revealed five subunits in *M. crystallinum* performing C3-photosynthesis in the well watered state with molecular masses of 69, 55, 41, 32 and 16 kDa. In the CAM state, seven subunits were detected with molecular masses of 69, 55, 41, 32, 31, 27 and 16 kDa [21]. The size of the polypeptide detected with anti TpP31Hv antibody in *M. crystallinum* protein extracts was about 31 kDa and therefore similar to the apparent molecular masses reported for other representatives of subunit E of the vacuolar ATPase [5,12–15]. In all studied species, the apparent molecular mass was by 5 to 6 kDa larger than the molecular mass deduced from the cDNA sequences. The apparent molecular mass of subunit E was identical in CAM and C3 plants. We therefore assume that the 32 kDa polypeptide, and not the 31 kDa subunit reported only for the CAM state, is the subunit E.

Salt stress has been shown to alter the activity of the vacuolar ATPase as well as the expression of the gene encoding subunit A [17,22–25]. In one report the amount of ATPase protein even decreased in cultured tobacco cells adapted to 428 mM NaCl, however, the proton pumping activity as related to ATPase protein increased [25]. This suggests enhanced capacity for ATP-driven transport by regulatory modulation of the protein. In all other studies, salt stress increased activity, message levels for subunit A or c, and protein amount of V-type ATPase by factors of

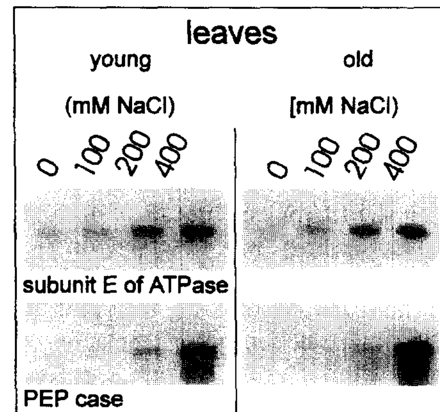

Fig. 4. TpP31 Mc-mRNA levels in leaves of *Mesembryanthemum crystallinum* plants in C3 or CAM mode. Plants were either treated with 0, 100, 200 or 400 mM NaCl in the irrigation water in order to induce CAM. For comparison, hybridization of duplicate blots was also performed with a cDNA encoding PEP carboxylase [20]. Plant RNA isolation, electrophoretic separation, transfer to Nylon membrane, synthesis of labeled probe and hybridisation were performed as described previously [5].

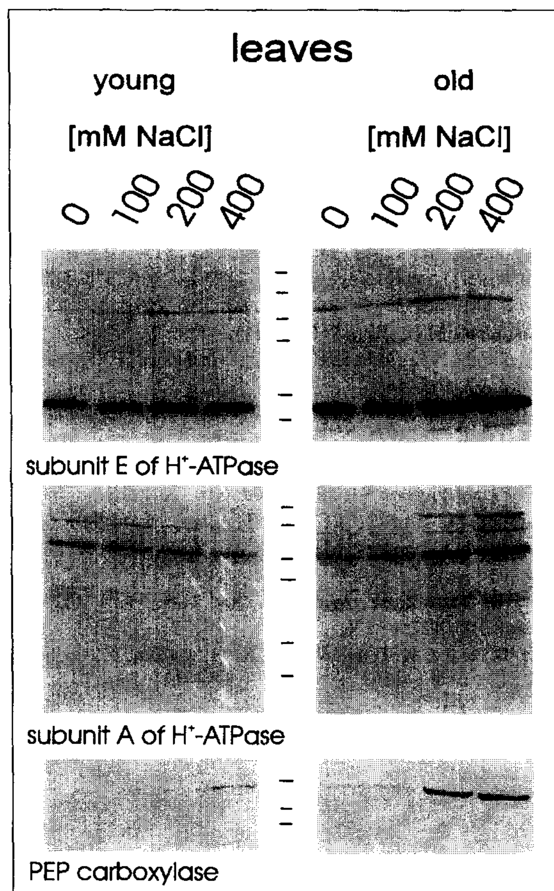

Fig. 3. Protein amounts of subunit E and A of the vacuolar ATPase, and of PEP carboxylase in leaves of *Mesembryanthemum crystallinum*. Proteins were extracted from young (left four lanes) or old leaves (right four lanes) and loaded in amounts equivalent to 33 mg fresh weight. The soil grown plants were either watered daily with NaCl-free water (lane 1 and 5), or with increasing NaCl concentrations: 100 mM: lane 2 and 6, 200 mM: lane 3 and 7; 400 mM: lane 4 and 8. Sizes of standard polypeptides are indicated. From the bottom, the lines indicate the position of the 84, 108 and 190 kDa marker polypeptides in the case of anti PEPCase, and 26.6, 36.5, 48.5, 58, 84 and 108 kDa in the case of anti-subunit E and A. Tissues were homogenized into buffer containing phenol for protein extraction followed by SDS-PAGE and Western blotting [5]. The antiserum was raised against the subunit E protein of barley. The preparation of an anti-PEPCase antibody has been described previously [28].

about 2 to 5. A similar increase is observed for subunit E protein in *Mesembryanthemum crystallinum* upon salt adaptation. The salt-induced response was stronger on the level of the mRNA than of the protein. This may indicate a higher turnover of ATPase subunits in plants performing CAM or under salt stress. Although Na accumulated in the leaves during short term experiments of 48 h duration, no rapid effects of salting was observed on mRNA- and protein-levels of subunit E which were unchanged within 48 h after initiation of the salt treatment (results not shown). Therefore, salt-dependent regulation of gene expression may be different for subunit A and subunit E of the vacuolar ATPase. It will be interesting to study the coordination of expression of the vacuolar ATPase-subunits in response to environmental stimuli.

In conclusion, subunit E must be considered to be a structurally highly conserved constitutive element of all vacuolar H^+-ATPases, whose expression is stimulated under growth conditions which require an increased pumping activity at the tonoplast for maintaining ion homeostasis in the plant cells.

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