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Molecular evidence for the occurrence of H^+-transporting V-ATPase subunit D and two different forms of subunit E in leaves of the obligate CAM species *Kalanchoë daigremontiana*

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Abstract

Membrane proteins of purified tonoplast vesicles from leaves of *Kalanchoë daigremontiana* Hamet et Perrier were solubilized by the non-ionic detergent Triton X-114 and subsequently separated by *MonoQ*® anion-exchange chromatography. Special attention was given to the range of molecular masses around 30 kDa comprising the central stalk subunit peptides of the H^+-transporting V-ATPase. Three polypeptides of apparent molecular masses of 32, 33 and 34 kDa were separated. Proteolytic fragments were obtained by trypsin digestion. Analysis by matrix-assisted laser desorption ionization (MALDI) mass spectrometry of tryptic fragments of the 32 and 33 kDa peptides and protein data-bank comparisons showed that they are two different forms of subunit E. N-terminal amino acid sequencing of tryptic fragments of the 34 kDa peptide showed that it is subunit D. This work provides for the first time unequivocal molecular evidence that the central stalk of the V-ATPase of the obligate CAM plant *K. daigremontiana* includes subunit D and different forms of subunit E.

Additional key words: crassulacean acid metabolism, proton pump, vacuole, tonoplast.

Introduction

The H^+-transporting vacuolar ATPase or V-ATPase, named after its occurrence in the tonoplast membrane of plant cell vacuoles, is an ubiquitous enzyme of membranes bordering acid compartments in plants, fungi and animals, *i.e.* membranes of vacuoles and vesicles and in some cases also the plasma membrane (Nelson 1995, Stevens and Forgac 1997, Ratajczak 2000). The holoenzyme is a very complex multi-subunit structure of two larger domains. The V_1-domain is separated into a central stalk and head region which protrudes from the membrane surface into the plasmatic phase. The V_o-domain is integral in the membrane. Using high resolution electron microscopy and sophisticated methods of image analysis, few years ago a second, peripheral stalk interconnecting the V_1-head and V_o has been identified (Boekema *et al.* 1997). Both domains and also the two regions of the V_1-domain are composed of several different polypeptides some of which have been ubiquitously found in all tissues studied, and hence, considered to be essential for function, some of which were only found in particular materials (for a review of the plant literature see Ratajczak 2000).

The central stalk region of the V_1-domain attaches the head region to the membrane integral V_o-domain and is likely to be part of the rotor of the V-ATPase holoenzyme together with the membrane-integral ring formed by

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Abbreviations: CAM - crassulacean acid metabolism; EGTA - ethyleneglycol-bis-(ß-aminoethylether)-N,N'-tetraacetic acid; EST - expressed sequence tag; HEPES - N-[2-hydroxyethyl] piperazine-N'-ethane-sulfonic acid; MALDI - matrix-assisted laser desorption ionization; SDS-PAGE - sodium dodecyl sulphate gel electrophoresis; SU - subunit; TOF - time of flight; V-ATPase - vacuolar-type H^+-tranlocating adenosine triphosphatase.

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several copies of subunit (SU) C. In the yeast and animal V-ATPase sequence information is available for SUs C, D and E which are suggested to be part of the central stalk (for review see Stevens and Forgac 1997). Molecular masses of proteins calculated from the nucleotide sequence of genes isolated from different organisms are 40 - 42 kDa (SU-C), 32 - 34 kDa (SU-D) and 27 - 33 kDa (SU-E) (for review see Stevens and Forgac 1997). For plant V-ATPases sequence information is available for central stalk subunits, *i.e.* SU-C of *Hordeum vulgare* (40 kDa; Tavakoli *et al.* 1999), SU-D of *Arabidopsis thaliana* (29.1 kDa; Kluge *et al.* 1999) and SU-E of *H. vulgare* (Dietz *et al.* 1995, Ford *et al.* 1997, direct protein data base submission, accession number AAD10336), *A. thaliana* and *Spinacia oleracea* (Dietz *et al.* 1996), *Mesembryanthemum crystallinum* (Dietz and Arbinger (1996), *Gossypium hirsutum* (Kim and Wilkins 1997a, direct protein data base submission, accession number O23948) and *Citrus limon* (Reuveni and Sadka 1999, direct protein data base submission, accession number AAD49706) with deduced molecular masses ranging from 26.2 to 27.1 kDa. Other SUs of the central stalk are the polypeptides D_i and E_i, with apparent molecular masses of 27-28 and 31-32 kDa, the N-terminal amino acid sequence of which has been determined by Bremberger and Lüttge (1992). These are induced in the facultative annual halophyte *M. crystallinum* during a salinity and age triggered shift from C_3-photosynthesis to crassulacean acid metabolism (CAM), where D_i is a proteolytic processing product of SU-B of the V-ATPase head (Zhigang *et al.* 1996). They have been suggested to stabilize the functional holoenzyme against perturbations (Ratajczak 1994, Lüttge *et al.* 1995).

Since the molecular masses of central stalk subunits D, E, D_i and E_i are very similar (see above), it is almost impossible to assign proteins separated on SDS-polyacrylamide gels to the respective subunits at the basis of apparent molecular mass comparison. However, in fact often the occurrence of subunits D and E was based only on circumstantial evidence of electrophoretic behaviour of peptides and evidence from protein-biochemistry and immunology. This included the V-ATPase of the obligatory CAM plant *Kalanchoë daigremontiana*, where a polypeptide of 32-33 kDa apparent molecular mass was detected in sodium dodecyl sulphate gel electrophoresis (SDS-PAGE) similar to SU-D of other V-ATPases and shown to belong to the V-ATPase by immunological evidence (Mariaux *et al.* 1994, Fischer-Schliebs *et al.* 1997), but where the evidence for the occurrence of SU-E (apparent molecular mass 28-33 kDa) was still more superficial since it was based only on comparison of apparent molecular masses (see Ratajczak 2000, and references therein).

Since the stalk subunits are so important not only in the housekeeping function of connecting the V-ATPase head to the V_o-domain in the membrane, but also in stress related perturbations and increased demands on vacuolar H^+-pumping, as for example in the salinity induced C_3-CAM shift in *M. crystallinum* (Bremberger and Lüttge 1992, Ratajczak *et al.* 1994), it appeared important to prove unequivocally that SUs D and E also occur in the obligate CAM plant *K. daigremontiana*. Since recent studies indicate that distinct isoforms of SU E exist in the *M. crystallinum* V-ATPase (Dietz and Arbinger 1996) and of SU D and SU E in the *Pisum sativum* V-ATPase (Kawamura *et al.* 2000), it is even more important to check the subunit composition of the V-ATPase holoenzyme at the protein level.

In a long-term program of characterizing tonoplast polypeptides of the molecular mass range around 30 kDa of CAM plants, which comprises the various V-ATPase stalk subunits (see above) as well as putative malate transporters of the tonoplast (Steiger *et al.* 1997), we developed a new solubilization and purification protocol. The non-ionic detergent Triton X-114 was used for solubilization since it was expected to solubilize preferentially hydrophobic membrane integral proteins. Our aim was to separate proteins around 30 kDa by anion-exchange chromatography and to identify these polypeptides by MALDI (matrix-assisted laser-desorption ionization) analysis and N-terminal amino acid sequencing of proteolytic fragments provided as subunits of the *K. daigremontiana* V-ATPase.

Materials and methods

Plants of *Kalanchoë daigremontiana* Hamet et Perrier were propagated vegetatively via adventitious plantlets from the collection of the Botanical Garden, Darmstadt. They were grown in Einheitserde (ED-73 DIN 11540-80T) under a day-length of at least 12 h extended to 14 h in winter by additional artificial illumination with *Philips* (Berlin, Germany) *SON-T AGRO 400* and *Osram* (Munich, Germany) *Scatto HQ-TS NDL* lamps [250 µmol(photon) m^{-2} s^{-1}; photosynthetically active radiation (400 - 700 nm)]. Leaves from the 3rd to 6th node from the apex were harvested at the end of the light period to minimize malic acid levels, stored over-night in air-tight bags in a coldroom at +4 °C and used the subsequent day for preparation of tonoplast vesicles.

Tonoplast vesicles were prepared according to Bremberger *et al.* (1988). Leaf slices were homogenized in a buffer containing 100 mM Tricine/Tris, pH 8.0, 3 mM $MgSO_4$, 3 mM ethyleneglycol-bis-(ß-amino-ethylether)-N,N'-tetraacetic acid (EGTA), 450 mM mannitol, 1 mM dithiothreitol and 0.5 % (m/v) polyvinyl-

pyrrolidone 40. After filtration and pre-centrifugation at 4 200 *g* for 10 min the microsomal fraction (supernatant) was subjected to a 90 min sucrose-cushion density ultracentrifugation at 100 000 *g*. The tonoplast-enriched fraction was collected from the 25 % (m/m) sucrose cushion, diluted with dilution buffer (10 mM N-[2-hydroxyethyl] piperazine-N'-ethane-sulfonic acid (HEPES), adjusted to pH 7.0 with NaOH, 3 mM $MgSO_4$, 1 mM dithiothreitol) and tonoplast vesicles were pelleted by 30 min centrifugation at 300 000 *g*. All steps were performed at +4 °C, the final pellet was resuspended in storage buffer (10 mM HEPES, adjusted to pH 7.0 with NaOH, 40 % (v/v) glycerol, 1 mM dithiothreitol, 3 mM $MgSO_4$), frozen immediately in liquid nitrogen and stored at -75 °C until use.

Purity of the membrane fraction was checked by measuring ATP-hydrolysis after Ames (1966) in the presence of various effectors of different membrane ATPases, namely 1 mM azide (inhibitor of mitochondrial F-ATPase; O'Neill *et al.* 1983), 40 nM bafilomycin A_1 (inhibitor of V-ATPase; Bowman *et al.* 1988, Dröse *et al.* 1993), 50 mM chloride (stimulator of V-ATPase; Jochem *et al.* 1984), 0.1 mM molybdate (inhibitor of non-specific phosphatases; Gallagher and Leonhard 1982), 50 mM nitrate (inhibitor of both mitochondrial F-ATPase and V-ATPase; O'Neill *et al.* 1983, Jochem *et al.* 1984). Protein contents were determined with amido-black after Popov *et al.* (1975) using bovine serum albumin as protein standard. Tonoplast-enrichment was 55-60 % of the membranes in fractions prepared.

Solubilization of membrane protein was performed using the nonionic detergent Triton X-114. Commercial Triton X-114 was purified removing contaminating more hydrophilic polyoxyethylene components according to Bordier (1981) using Tris-buffered NaCl (10 mM Tris/HCl, pH 7.5, 100 mM NaCl). Solubilization followed McIntosh and Oliver (1992) in a solubilization buffer of 10 mM HEPES/Tris, pH 7.5, 3 mM $MgSO_4$, 1 mM dithiothreitol, 40 % (v/v) glycerol and 3 % (m/v) Triton X-114 with 2 - 3 mg tonoplast protein in a volume of 1.35 cm^3. The mixture was incubated for 30 min on ice with repeated mixing using a *Vortex* and then centrifuged for 35 min at +4 °C and 100 000 *g*. The solubilisate supernatant gave two phases of different density (Table 1).

Table 1. Tonoplast protein balance after solubilization with Triton X-114 reagent. Replicates of protein analyses in the fractions $n = 11$ - 13, errors are given as $SD_{(n-1)}$.

Fraction	Protein content [mg fraction^{-1}]	[% of initial]
Vesicle suspension	2.52 ± 0.40	100
S1 supernatant	1.29 ± 0.17	51
S2 supernatant	0.77 ± 0.24	30
Pellet	0.47 ± 0.11	19

For separation of polypeptides via *Pharmacia* (Freiburg, Germany) *MonoQ®-FPLC* (fast-protein-liquid-chromatography) the strong anion-exchanger column *HR 5/5* (*Pharmacia*, Freiburg, Germany) was used. Separation buffer was 20 mM 1,3-bis-[tris-(hydroxymethyl)-aminomethane]-propane/HCl, pH 7.0, 4 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM EGTA, 1 mM ethylenediamine-tetraacetic acid, 0.1 % (m/v) Triton X-100. Flow rate was 0.5 cm^3 min^{-1}. Solubilized proteins were applied in a volume of 1 cm^3. After washing the column with 20 cm^3 separation buffer a linear gradient of 0 - 300 mM KCl in separation buffer was used for elution. Tightly binding proteins were then eluted with 1 mM KCl in the separation buffer. Volume of fractions collected was 1 cm^3.

Before SDS-PAGE proteins were concentrated and cleaned from the remaining detergent by precipitation with trichloro-acetic acid and washing of the precipitate with acetone (Serrano 1988, Rehm 1997). After evaporating the acetone, the proteins were dissolved in a buffer with 62.5 mM Tris/HCl, pH 6.8, 8.7 % (v/v) glycerol, 3 % (v/v) β-mercaptoethanol, 2 % (m/v) SDS and 0.01 % (m/v) bromo-phenol blue.

SDS-PAGE was performed using gel systems according to Laemmli (1970) and Schägger and von Jagow (1987), respectively. In the separation gel the total acrylamide concentration and the concentration of bisacrylamide related to total acrylamide ratio were 17.0 and 3.3 % for Laemmli gels and 16.5 and 3.0 % for Schägger and von Jagow gels, respectively. Molecular mass standard kits used in SDS-PAGE were LMW-standard and the Kaleidoscope prestained standard of *BioRad* (Munich, Germany). Proteins separated in the gels were silver-stained after Merill *et al.* (1981) or Coomassie-stained after Neuhoff *et al.* (1988).

Immunoprecipitation was performed as described previously using antisera against the V-ATPase holoenzyme of *K. daigremontiana* subsequently called *ATP95* (Fischer-Schliebs *et al.* 1997) and against subunit A of the V-ATPase head of *M. crystallinum* subsequently called *anti-A* (Ratajczak *et al.* 1994) coupled to protein A-sepharose (*Sigma*, Deisenhofen, Germany).

Proteolytic polypeptide fragments for N-terminal amino acid sequencing and for MALDI (matrix-assisted laser-desorption ionization) analyses were obtained *via* digestion with 0.5 g dm^{-3} sequence grade modified porcine trypsin (*Promega*, Madison, USA) in 40 mM ammonium bicarbonate, pH 8.4, at 37 °C overnight.

In MALDI analysis polypeptide fragments are ionized by a laser pulse in a high vacuum and then separated according to their mass/charge (m/z) ratio in a time-of-flight (TOF) mass spectrometer. Analyses were performed using a 337 nm nitrogen laser and a reflector-*TOF Reflex II* (*Bruker-Daltonik*, Bremen, Germany). Individual laser shots (50 to 200) were used to improve signal/noise ratio. A two-point mass-calibration was obtained using the autolysis products of trypsin at

m/z 842.5 and at m/z 2211.1 as internal standard ions. Samples were applied with a thin-layer preparation technique with 0.3 mm^3 of a saturated solution of α-cyano-4-hydroxy-cinnamic acid in acetone as matrix substance, subsequently applying 0.8 mm^3 10 % (m/v) formic acid and 0.4 mm^3 trypsin digested protein solution. To remove salts of the digestion buffer the spots were washed with 10 % (m/v) formic acid and bi-distilled water. The MALDI-spectra provide very precise molecular masses of peptide fragments which were compared with different computer search-programs (*MS-Fit*, *Pro-Found*, *Peptide-Search*) at a mass precision of 0.1 Da.

Results

In the supernatant (S) of the solubilization assay two phases were separated, a larger somewhat greenish one (S1) with lower density and a smaller colorless and somewhat more viscous one (S2) (Table 1). When commercial Triton X-114 is purified the detergent phase is the phase with the higher density below the aqueous phase. Therefore, the denser S2 phase was used for further purification by anion-exchange chromatography (*MonoQ®-FPLC*).

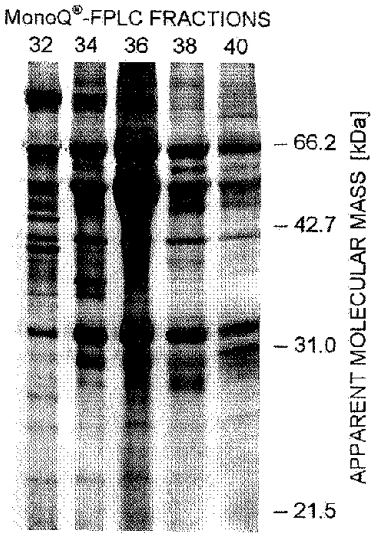

Fig. 1. Silver-stained SDS-PAG electrophoregramme after Laemmli (1970) of *MonoQ®-FPLC* fractions 32 - 40. Apparent molecular masses were derived from a LMW marker standard (not shown).

Elution of proteins from the *MonoQ®* anion-exchange column by separation buffer with increasing KCl-concentrations was followed via monitoring absorption at 280 nm. Peaks occurred in fractions 30 (*ca.* 150 mM KCl) and 36 (*ca.* 240 mM KCl); more tightly bound proteins eluted at 1 M KCl. Protein patterns of these *MonoQ®* fractions were tested by SDS-PAGE. In the range of apparent molecular masses around 30 kDa, which was of most interest in the present study, heavily stained bands were concentrated in fractions 32 - 40 (Fig. 1). To obtain a better separation of polypeptides in this range of molecular masses than provided by the Laemmli (1970) gel (Fig. 1), a different gel was used according to Schägger and von Jagow (1987) for further examination of the MonoQ® fractions 35 and 36 (Fig. 2). This revealed 3 clearly separated bands of apparent molecular masses of 34, 33 and 32 kDa subsequently referred to as MQ34, MQ33 and MQ32.

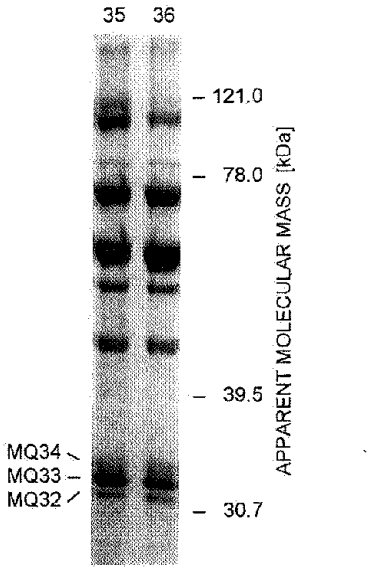

Fig. 2. Coomassie brilliant blue-stained SDS-PAG electrophoregramme after Schägger and von Jagow (1987) of *MonoQ®-FPLC* fractions 35 and 36 (numbers on the top of traces) and a Kaleidoscope marker kit (not shown) of given molecular masses. The peptides MQ34, MQ33 and MQ32 are indicated on the left-hand margin.

To test whether these polypeptides were part of the V-ATPase holoenzyme complex an immunoprecipitation was performed using antisera directed against the V-ATPase holoenzyme of *K. daigremontiana* (*ATP95*) and subunit A (*anti-A*) of *M. crystallinum*, respectively (Fig. 3). Indeed, the typical V-ATPase-peptide band-pattern was obtained in the pellets of immuno-precipitation assays (P35 and P37 in Fig. 3) although immunoprecipitation was not complete (see S35 and S37 in Fig. 3). The interesting peptides in the 30 kDa range ("D?" in Fig. 3) were also pelleted in immuno-precipitation. Hence, it appeared warranted to subject these bands to MALDI analysis and N-terminal amino

acid sequencing in the quest for SUs D and E of *K. daigremontiana*.

After proteolytic breakdown by trypsin digestion 10 of the detected fragments of MQ32 and MQ33 showed molecular masses identical to proteolytic breakdown fragments of V-ATPase SU-E of *M. crystallinum*, while a

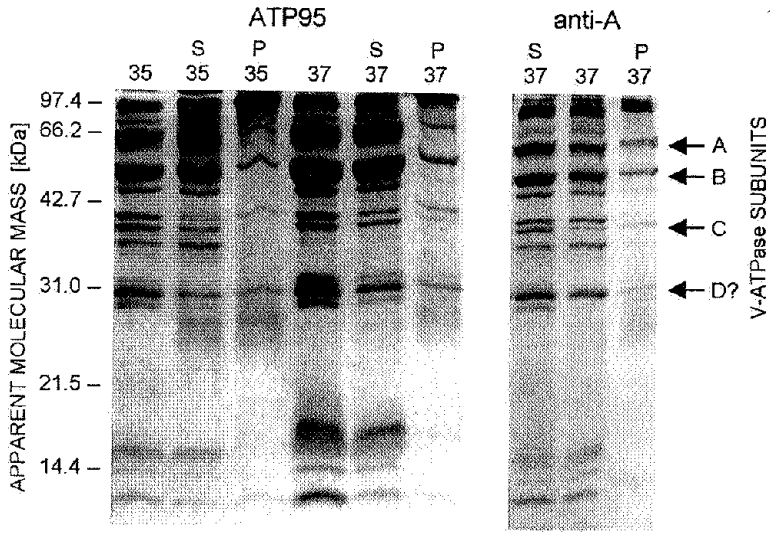

Fig. 3. Coomassie brilliant blue-stained SDS-PAG electrophoregramme after Schägger and von Jagow (1987) of *MonoQ*®*-FPLC* fractions 35 and 37 (numbers on top of traces) and supernatants (S) as well as pellets (P) of immunoprecipitations of the respective fractions using *ATP95* and *anti-A* antisera, respectively. Apparent molecular masses were derived from an LMW marker standard (not shown). Putative V-ATPase subunits are indicated by letters.

Table 2. Molecular masses of proteolytic fragments of of MQ32 and MQ33 of *K. daigremontiana*. Mass of the fragment plus the mass of a bound proton is compared with protein data-base information of V-ATPase subunit E from different other plant species (*M.c.* - *Mesembryanthemum crystallinum* (Dietz and Arbinger 1996 [Acc.-No. Q40272]); *C.l.* - *Citrus limon* (Reuveni and Sadka 1999, direct protein data base submission [Acc.-No. AAD49706]); *H.v.* - *Hordeum vulgare* (Ford *et al.* 1997, direct protein data base submission [Acc.-No. AAD10336]); *Z.m.* - *Zea mays* (Stapleton and Walbot 1999, direct protein data base submission [Acc.-No. AAD45282]); *S.o.* - *Spinacia oleracea* (Dietz *et al.* 1996 [Acc.-No. CAA65581]); *G.h.* - *Gossypium hirsutum* (Kim and Wilkins 1997b [Acc.-No. AAB72177]); *A.t.* - *Arabidopsis thaliana* (Bevan *et al.* 2000, direct protein data base submission [Acc.-No. CAB43050]). Amino acid positions and amino acid sequences refer to the amino acid sequence of *M.c.* SU-E with the exception of fragment 9 which refers to *H.v.* SU-E. Asterisks indicate the identity of molecular mass of proteolytic fragments of MQ32, MQ33 of *K. daigremontiana* and of subunit E from the other species at a mass precision of 0.1 Da; n.d., not determined since this fragment is not present in the expressed sequence tag clone of *Z.m.* (Stapleton and Walbot 1999, direct protein data base submission [Acc.-No. AAD45282]). Amino acids in brackets indicate the trypsin cleavage sites.

MQ32 MQ33	Molecular mass [Da]	Amino acid position	Amino acid sequence	*M.c.* Q 40272	*C.l.* AAD 49706	*H.v.* AAD 10336	*Z.m.* AAD 45282	*S.o.* CAA 65581	*G.h.* AAB 72177	*A.t.* CAB 43050
1	724.31	53 - 57	(R)QEYER(K)	*	*	*	*			
2	754.49	215 - 220	(K)KLPQIR(K)	*			n.d.	*		
		216 - 221	(K)LPQIRK(Q)	*			n.d.	*		
3	929.49	41 - 48	(K)LQLVEAEK(K)	*	*	*	*	*	*	*
4	993.49	51 - 57	(K)IRQEYER(K)	*	*	*	*			
5	1038.60	125 - 133	(R)LKEPAVLLR(C)	*	*	*	n.d.			
6	1056.62	127 - 135	(K)EPAVLLRCR(E)	*	*	*	n.d.			
7	1311.63	68 - 78	(K)IEYSMQLNASR(I)	*	*	*	*	*	*	
8	1439.75	67 - 78	(R)KIEYSMQLNASR(I)	*	*	*	*	*	*	
9	1483.77	79 - 91	(R)IKVLQAQDDLVNK(M)			*	*			
10	1595.82	66 - 78	(R)RKIEYSMQLNASR(I)	*				*		
11	1808.88	25 - 40	(K)ANEISVSAEEEFNIEK(L)	*	*		*		*	*

Table 3. N-terminal amino acid sequences of two proteolytic fragments of MQ34 (MQ34-1 and MQ34-2). Sequences are compared with protein data-bank information (SU-D=D subunit of the V-ATPase). Asterisks indicate identical amino acids. References: 1, Kluge *et al.* (1999), Acc.-No. CAB46439; 2, Nahm *et al.* (1998), direct protein data base submission, Acc.-No. AA751826; 3, Nelson *et al.* (1995), Acc.-No. P39942; 4, Sakai *et al.* (1995), Acc.-No. AAD10366; 5, Janbon *et al.* (1997), direct protein data base submission, Acc.-No. P87220; 6, Margolles-Clarke and Bowman (1998), direct protein data base submission, Acc.-No. O59941; 7, Mulligan *et al.* (1993), direct protein data base submission, Acc.-No. P32610; 8, Wilson *et al.* (1994), Acc.-No. P34462; 9, Bult *et al.* (1996), Acc.-No. Q58032; 10, Smith *et al.* (1997), Acc.-No. AAB85449.

Polypeptide fragment	N-terminal amino acid sequence of fragments									
MQ34-1	K	S	D	A	L	T	V	Q	F	R
A. thaliana SU-D[1]	*	*	*	*	*	*	*	*	*	*
EST from *Oryza sativa*, AA751826[2]	*	*	*	*	*	*	*	*	*	*
Bos taurus SU-D[3]	*	*	*	*	*	*	L	R	*	*
Oryctolagus cuniculus SU-D[4]	*	*	*	*	*	*	L	R	*	*
Candida albicans SU-D[5]	*	*	E	*	*	*	K	R	*	*
Neurospora crassa SU-D[6]	*	*	E	*	*	*	K	R	*	*
Saccharomyces cerevisiae SU-D[7]	*	*	E	*	*	*	K	R	*	*
Caenorhabditis elegans SU-D[8]	*	A	*	*	*	N	L	R	*	*
Methanococcus jannaschii SU-D[9]	*	R	*	*	*	I	M	E	*	F
Methanobacterium thermoautotrophicum SU-D[10]	*	R	N	*	*	I	M	E	*	F
MQ34-2	D	S	S	F	A	L	T	E	V	K
A. thaliana SU-D[1]	T	*	*	*	*	*	*	*	*	*
Bos taurus SU-D[3]	E	A	A	*	S	*	A	*	A	*
Oryctolagus cuniculus SU-D[4]	E	A	A	*	S	*	A	*	A	*
Candida albicans SU-D[5]	T	A	A	*	S	*	A	*	*	Q
Neurospora crassa SU-D[6]	I	A	*	L	S	*	A	*	*	T
Saccharomyces cerevisiae SU-D[7]	T	A	A	*	S	*	A	*	*	S
Caenorhabditis elegans SU-D[8]	E	A	A	*	S	*	A	*	A	*
Methanococcus jannaschii SU-D[9]	E	A	Y	K	D	*	I	M	A	Q
Methanobacterium thermoautotrophicum SU-D[10]	E	A	F	S	D	*	*	*	A	Q

smaller number of matches was obtained for SUs-E from other plant species (Table 2).

Identification of MQ34 proved impossible via MALDI analysis of proteolytic fragments and subsequent database searches with the masses obtained. However, N-terminal amino acid sequencing of two fragments of MQ34 was successful (Table 3). The first one, MQ34-1 showed 100 % identity in 10 subsequent amino acids of *A. thaliana* SU-D (Kluge *et al.* 1999) and of an amino acid sequence deduced from an EST (expressed sequence tag)-clone isolated from rice (*Oryza sativa*). The amino acid sequence of MQ34-1 matches the sequence of the *A. thaliana* SU-D from amino acids 37 - 46. Sequence identity of 80 % in 10 subsequent amino acids of animal SU-D, namely bovine (*Bos taurus*) and rabbit (*Oryctolagus cuniculus*), was detected, while sequence similarity with SU-D from other non-plant species was lower (see Table 3). The second one, MQ34-2, showed 90 % identity in 10 subsequent amino acids of SU-D from *A. thaliana* (amino acids 65 - 74) while sequence similarity with SU-D from non-plant species was lower (Table 3).

Discussion

Among Triton X-114 solubilized tonoplast proteins of *K. daigremontiana* three polypeptides of apparent molecular masses of 32, 33 and 34 kDa could be clearly separated by anion-exchange chromatography (*MonoQ*^{R}-*FPLC*) and were denominated MQ32, MQ33 and MQ34. MALDI analysis of proteolytic fragments of both MQ32 and MQ33 and protein data-bank comparisons identified these two polypeptides as V-ATPase subunit E (SU-E). N-terminal amino acid sequencing of proteolytic fragments identified MQ34 as V-ATPase subunit D (SU-D).

For the *K. daigremontiana* V-ATPase due to its electrophoretic behaviour and due to immunological evidence a 32 - 33 kDa polypeptide was suggested to be subunit D (Mariaux *et al.* 1994, Fischer-Schliebs *et al.* 1997). As mentioned above (see Introduction) up to now an SU-E like polypeptide has only been assumed to occur in *K. daigremontiana* on the basis of apparent molecular mass comparison with SUs-E from other species. MALDI analyses and partial amino acid sequencing performed in the present study for the first time confirm unequivocally

that both subunits D and E are genuine subunits of the V-ATPase of *K. daigremontiana*, and that the V-ATPase holoenzyme is complete with respect to these two important subunits of the central stalk.

This is an essential observation. While the unequivocal identification of SU-D now is reassuring confirmation of the more circumstantial evidence from immunological investigations, the uncertainty of the presence of SU-E in *K. daigremontiana* was quite intriguing. SU-E was sequenced and cloned in both the C_3-plants barley (*H. vulgare*) (Dietz *et al.* 1995, Ford *et al.* 1997, direct protein data base submission, accession number AAD10336), mouse-ear cress (*A. thaliana*) (Dietz *et al.* 1996), spinach (*Spinacia oleracea*) (Dietz *et al.* 1996) and lemon (*Citrus limon*) (Reuveni and Sadka 1999, direct protein data base submission, accession number AAD49706) and the facultative halophyte and C_3/CAM-intermediate *M. crystallinum* (Dietz and Arbinger 1996). Why should the V-ATPase in the obligate CAM species *K. daigremontiana* be able to operate without a central stalk subunit E? This was particularly puzzling since the central stalk subunits are well known to be important for stabilizing the V-ATPase especially under strong demand of H^+-pumping as given by nocturnal vacuolar malic acid accumulation in CAM. In *M. crystallinum* even two additional central stalk subunits are formed during salinity-induced transition from C_3-photosynthesis to CAM, namely subunits D_i and E_i (Bremberger and Lüttge 1992, Ratajczak *et al.* 1994), which has been shown to add to stability of the polypeptide complex of the V-ATPase (Ratajczak 1994, Lüttge *et al.* 1995).

That we detected two polypeptides with slightly different apparent molecular masses, MQ32 (32 kDa) and MQ33 (33 kDa) which both were identified as SU-E can be explained in 3 different ways: *1*) the original SU-E polypeptide could have been modified in different ways during the isolation process; *2*) MQ32 could be a proteolytic digestion product of MQ33; *3*) MQ32 and MQ33 could be different SU-E isoforms. In terms of function the latter, of course, would be the most interesting possibility, but this cannot be decided without much more detailed characterization of the two polypeptides than was possible in the present study.

In conclusion, the demonstration of the occurrence of both SU-D and SU-E by the present protein-biochemical analysis of the tonoplast of *K. daigremontiana* underlines the ubiquity of SU-E. It also emphasizes the requirement of the complete central stalk subunit complement for functioning of the V-ATPase in this obligatory CAM plant. Future studies have to show whether V-ATPases containing different forms of SU-E exhibit distinct enzymatic properties.

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