ELSEVIER

Biochimica et Biophysica Acta 1564 (2002) 243–255

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

Phenotypic subunit composition of the tobacco (*Nicotiana tabacum* L.) vacuolar-type H^+-translocating ATPase

Martina Drobny [a], Martina Schnölzer [b], Sabine Fiedler [b], Ulrich Lüttge [a], Elke Fischer-Schliebs [a], Anna-Luise Christian [a], Rafael Ratajczak [a,*]

[a] *Darmstadt University of Technology, Institute of Botany, Schnittspahnstraße 3-5, D-64287, Darmstadt, Germany*
[b] *German Cancer Research Center, Protein Analysis Facility, Im Neuenheimer Feld 280, D-69120, Heidelberg, Germany*

Received 17 January 2002; received in revised form 15 April 2002; accepted 22 April 2002

Abstract

The model plant tobacco (*Nicotiana tabacum* L.) was chosen for a survey of the subunit composition of the V-ATPase at the protein level. V-ATPase was purified from tobacco leaf cell tonoplasts by solubilization with the nonionic detergent Triton X-100 and immunoprecipitation. In the purified fraction 12 proteins were present. By matrix-assisted laser-desorption ionization mass spectrometry (MALDI-MS) and amino acid sequencing 11 of these polypeptides could be identified as subunits *A*, *B*, *C*, *D*, *F*, *G*, *c*, *d* and three different isoforms of subunit *E*. The polypeptide which could not be identified by MALDI analysis might represent subunit *H*. The data presented here, for the first time, enable an unequivocal identification of V-ATPase subunits after gel electrophoresis and open the possibility to assign changes in polypeptide composition to variations in respective V-ATPase subunits occurring as a response to environmental conditions or during plant development.

Keywords: MALDI analysis; Protein sequencing; Subunit; Tonoplast; V-ATPase; Tobacco (*Nicotiana tabacum* L.)

1. Introduction

Compartmentation by and regulation of ion and metabolite fluxes across biological membranes are prerequisite for all living organisms. Selective permeability of biological membranes is caused by a variety of integral membrane transport proteins, which can be divided into distinct groups, i.e. passive, primary-active and secondary-active transport systems. The primary-active transporters can be considered as master transport systems, since they energize the membranes by the establishment of electrochemical ion or proton gradients, utilizing the energy released during cleavage of energy-rich phosphodiester bonds of adenosine triphosphate (ATP) or inorganic pyrophosphate. At the tonoplast, the membrane surrounding the large central vacuole of plant cells, the vacuolar-type H^+-translocating ATPase (V-ATPase; EC 3.6.1.34; for reviews see Refs. [1–3]) is the main primary-active transporter, energizing the membrane for ion and metabolite transport by establishing an electrochemical proton gradient.

The V-ATPase holoenzyme consists of two domains, i.e. the integral membrane domain V_o and the membrane peripheral domain V_1 [4–6]. The V_1 domain itself is a 'head and stalk' structure. In addition to the long-known central stalk, connecting the head with V_o, recently a second peripheral stalk has been detected [7,8], which is suggested to be a prerequisite structural feature for coupling ATP hydrolysis with proton transport according to the rotor-stator model of F-type ATP synthase and V-ATPase function [9,10]. In yeast (*Saccharomyces cerevisiae*) 13 genes have been identified encoding for V-ATPase subunits. These subunits seem to be present in the V-ATPase holoenzyme in a stoichiometry of $A_3B_3CDEFGHac_6c'\,c''d$ (capital and small letters indicate subunits of V_1 and V_o, respectively; [11]). Not all genes homologous to the yeast V-ATPase genes have been characterized in plant genomes so far. Even after complete sequencing of the *Arabidopsis thaliana* genome there is only evidence for the existence of subunits *A*, *B*, *C*, *D*, *E*, *F*, *G*, *H*, *a*, *c* and *d*, subunit *c″* might be present, while subunit *c'* is missing [12]. At the protein level, in purified V-ATPase fractions from different plant

* Corresponding author. Present address: Deutscher Akademischer Austauschdienst (DAAD), Kennedyallee 50, D-53175, Bonn, Germany. Tel.: +49-228-882-339; fax: +49-228-882-551.
E-mail address: r.ratajczak@daad.de (R. Ratajczak).

0005-2736/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0005-2736(02)00459-5

species up to 10 distinct polypeptides have been found (*Avena sativa* [13], *Hordeum vulgare* [14], *Pyrus communis* [15]).

Studies on the plant V-ATPase revealed that V-ATPase amount, subunit composition and subunit stoichiometry seem to vary in different types of tissue, in response to environmental factors and due to the developmental state of the plant (for reviews see Refs. [1,3]. Everybody investigating the plant V-ATPase at the protein level knows how difficult it is to assign unequivocally a polypeptide of a partially purified V-ATPase fraction to a V-ATPase subunit. The range of apparent molecular masses reported for individual V-ATPase subunits from different plant species spans up to 20 kDa, e.g. 95–115 kDa for subunit *a*, 63–72 kDa for subunit *A*, 52–60 kDa for subunit *B*, 37–52 kDa for subunit *C*, 32–36 kDa for subunit *d*, 30–42 kDa for subunit *D* and 27–32 kDa for subunit *E* (for review see Ref. [2]). It is obvious that the molecular mass ranges for subunits *B*, *C*, *d*, *D* and *E* are overlapping. Thus, it is highly risky to assign polypeptides to V-ATPase subunits simply by apparent molecular mass comparison. One can imagine that it is almost impossible to draw conclusions about environmental and developmental changes of respective V-ATPase subunits if these subunits are not unequivocally identified.

Even the use of antibodies for identification of V-ATPase subunits might not be helpful in all cases for the following reasons. First, most antibodies used show immunological cross-reactions with other proteins and, second, in most cases the antigen for antibody production has been selected by comparison of the apparent molecular mass of a purified polypeptide with the predicted molecular mass of a V-ATPase subunit. An unequivocal identification of a polypeptide is only possible by comparison of its amino acid sequence with the amino acid sequence deduced from the nucleotide sequence of a V-ATPase gene.

The aim of the present study was to identify the subunit composition of a plant V-ATPase at the protein, i.e. at the phenotypic level, using the methods of Edman degradation for determination of partial amino acid sequences and matrix-assisted laser-desorption ionization mass spectrometry (MALDI-MS). It has to be mentioned that such a survey at present is not only missing for the plant V-ATPase but also for the yeast and animal V-ATPases, which are much better characterized at the level of genes. We decided to use tobacco (*Nicotiana tabacum* L.) as a model plant for our survey. Due to its high economical importance and since it can easily be genetically transformed by the *Agrobacterium tumefaciens* system, tobacco became a model plant in physiological and cytological research. Using tobacco a variety of different studies have been performed, only some examples of which can be mentioned here, e.g. studies on vacuolar proteins [16–21], on salinity and osmotic stress [22–24], on amino acid and sugar transport and metabolism [25], on metabolite transport at the tonoplast in relation to nutrient nutrition [26], on cell cycle [27–29], on oxidative stress [30–32] and on phytohormones [33,34]. By application of different MALDI-MS techniques and amino acid sequencing, we identified polypeptides present in a tobacco V-ATPase fraction purified by immunoprecipitation as V-ATPase subunits.

2. Materials and methods

2.1. Plant material and isolation of a tonoplast-enriched membrane vesicle fraction

Plants of tobacco (*N. tabacum* L.) cv. Samsun N.N. were cultivated under a 12 h light (photosynthetically active radiation (400–700 nm) 200–300 μmol photons m^{-2} s^{-1} at plant level):12 h dark regime at 28 °C and 60% relative humidity during the day and 20 °C and 80% relative humidity during the night. Plants were cultivated in sand and were supplied daily to field capacity with nutrient solution according to Johnson et al. [35] containing 10 mM $Ca(NO_3)_2$ as the only nitrogen source. At the time of harvest plants were 7–8 weeks old.

A tonoplast-enriched membrane vesicle fraction was isolated from leaf homogenates by sucrose cushion density centrifugation as described in Ref. [36].

2.2. Purification of the V-ATPase by immunoprecipitation

Immunoprecipitation of the V-ATPase from 150 μg tonoplast proteins solubilized for 30 min in 1% (w/v) Triton X-100 at 4 °C was performed as described previously [19] using an antiserum against subunit *A* of the V-ATPase head of *Mesembryanthemum crystallinum* [37], subsequently called *anti-A*, coupled to protein A-sepharose (Sigma, Deisenhofen, Germany). After incubation of solubilized tonoplast proteins with *anti-A*/protein A-sepharose for 1 h at 4 °C and centrifugation for 10 min at 12,000 × *g* the supernatant was discarded. The pellet (immunoprecipitate) was washed twice with phosphate buffered saline containing 1% (w/v) Triton X-100.

2.3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot

SDS-PAGE was performed using the gel system according to Schägger and von Jagow [38]. In the separation gel the total acrylamide concentration was 16.5% (w/v) and the concentration of bisacrylamide related to total acrylamide ratio was 3.0%. Molecular mass standard kits used in SDS-PAGE were LMW-standard and the Kaleidoscope prestained standard purchased from BioRad (Munich, Germany). Proteins separated in the gels were Coomassie-stained after Schägger and von Jagow [38].

Western blot of proteins from polyacrylamide gels to PVDF membranes (Immobilon P, Millipore, Eschborn, Germany) and immuno-staining were performed as described by Fischer-Schliebs et al. [19].

2.4. MALDI-MS and amino acid sequence analysis

Proteolytic polypeptide fragments for N-terminal amino acid sequencing and for MALDI analysis were obtained via digestion with 0.5 μg μl^{-1} sequence grade modified porcine trypsin (Promega, Madison, WI, USA) in 40 mM ammonium bicarbonate, pH 8.4, at 37 °C overnight.

In MALDI-MS 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 with delayed extraction using a 337-nm nitrogen laser and a reflector-TOF Reflex II (Bruker-Daltonik, Bremen, Germany). Ion acceleration voltage was set to 20.0 kV, the reflector voltage was 21.5 kV and the first extraction plate was set to 15.7 kV. Individual laser shots (50 to 200) were used to improve the signal/noise ratio. A two-point mass-calibration was obtained using the autolysis products of trypsin at protonated molecular mass values of *m*/*z* 842.50 and *m*/*z* 2211.10 as internal standard ions. Samples were applied using a thin-layer preparation technique with 0.3 μl of a saturated solution of α-cyano-4-hydroxy-cinnamic acid in acetone as matrix substance, subsequently applying 0.8 μl 10% (w/v) formic acid and 0.4 μl trypsin digested protein solution. To remove salts of the digestion buffer the spots were washed with 10% (w/v) formic acid and bi-distilled water. A detection window ranging from *m*/*z* 700 to *m*/*z* 2800 was used in all experiments. The MALDI spectra provided 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. In the present study only the MALDI spectrum obtained for the total tryptic digest of the polypeptide *N.t.*-16 is shown as an example (see Fig. 3).

For MALDI fragment ion analysis (post-source decay fragmentation, PSD) the proteolytically cleaved protein sample was desalted using $ZipTip_{C18}$ pipette tips (Millipore) and directly eluted onto the target with 1 μl of a saturated solution of α-cyano-4-hydroxy cinnamic acid in 50% acetonitrile:50% H_2O. The fragment ion spectrum of the proteolysis product giving the largest signal in the MALDI spectrum was recorded using an ion selector (Fast Pulser). The molecular masses of fragment ions were used for data base searches with the program MS-Tag (Protein Prospector Program Package) at a mass precision of 0.5 Da for the fragment ions and 0.1 Da for the parent ion.

Amino-acid sequence analysis of proteolytic protein fragments after Edman [39] was performed with an Edman sequencer (Procise 494 cLC gas phase sequencer, Applied Biosystems, Weiterstadt, Germany). Prior to sequencing protein fragments were separated by capillary HPLC using a BDS-Hypersil C_{18} column (150 mm × 300 μm; LC-Packings, Amsterdam, The Netherlands) at a flow rate of 4 ml/min in 0.1% (w/v) trifluoro acetic acid in an increasing acetonitrile concentration gradient from 10% to 60% (v/v) for 90 min.

2.5. Determination of protein concentration

Protein concentration was determined by the Amidoblack 10^B method described by Popov et al. [40] using bovine serum albumin as a standard.

3. Results

For the identification of V-ATPase subunits by amino-acid sequencing or MALDI analysis it is prerequisite to isolate highly purified V-ATPase as starting material. Thus, V-ATPase was purified in a single-step procedure by immunoprecipitation from Triton X-100 solubilized tonoplast proteins using an antiserum directed against subunit *A* of the *M. crystallinum* V-ATPase coupled to protein A-sepharose. In this way the entire V-ATPase holoenzyme binds to sepharose-coupled protein A via the antibody and can be separated from other solubilized tonoplast proteins by differential centrifugation. Immuno-decoration of tobacco tonoplast polypeptides with *anti-A* after Western blot analysis resulted in staining of a single polypeptide exhibiting a molecular mass of 64 kDa and shows the specificity of the antiserum used for V-ATPase immunoprecipitation (Fig. 1, lane 1). A comparison of the poly-

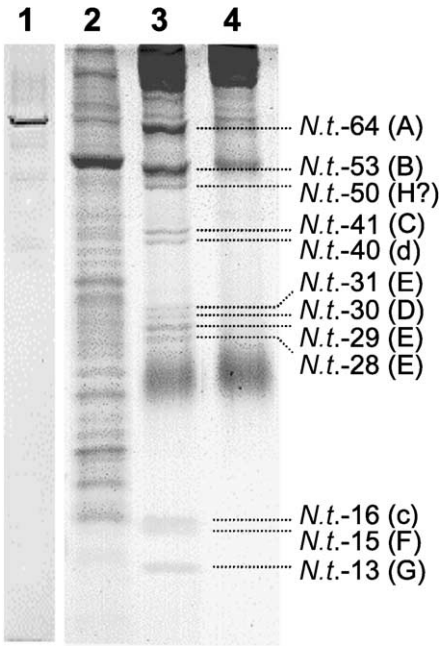

Fig. 1. Western blot analysis (lane 1) and a Coomassie brilliant blue-stained SDS-polyacrylamide gel (16.5% acrylamide) (lanes 2–4) showing the cross-reaction of tobacco tonoplast proteins with the antiserum *anti-A* directed against V-ATPase subunit *A* of *M. crystallinum* (lane 1) and the polypeptide compositions of a tobacco tonoplast vesicle fraction (lane 2), immunoprecipitated tobacco V-ATPase (lane 3) and the IgG/protein A-sepharose complex used for immunoprecipitation as a control (lane 4). On the right-hand margin tobacco (*N.t.*) polypeptides assumed to be subunits of the V-ATPase are indicated and denominated according to their apparent molecular mass. Designation to V-ATPase subunits is given in brackets.

peptide composition of the tobacco tonoplast protein fraction (Fig. 1; lane 2) with the immunoprecipitated fraction (Fig. 1; lane 3) underlines the effectiveness of the method. Besides several polypeptides which represent constituents of the IgG/protein A complex (see Fig. 1, lane 4), only 12 polypeptides were visible, which were denominated *N.t.*-(for *N. tabacum*) 64, 53, 50, 41, 40, 31, 30, 29, 28, 16, 15 and 13 according to their apparent molecular mass. The identity of these polypeptides was analyzed by amino acid sequencing and/or MALDI analysis to assess whether they are subunits of the tobacco V-ATPase and which subunits they represent. In the following paragraphs data obtained will be presented according to the apparent molecular mass of the detected polypeptides starting with the largest one.

According to its apparent molecular mass, *N.t.*-64 most probably represents subunit *A*. This was verified by MALDI-MS and a data base search using the protonated masses of the detected tryptic fragments of *N.t.*-64 found in the detection window of *m*/*z* 700–2800 (Table 1). Since the amino acid sequence of the tobacco V-ATPase subunit *A* is unknown, the data had to be compared with *A*-subunits from other plant species. All eight tryptic fragments of *N.t.*-64 found could be assigned to theoretical tryptic fragments of subunit *A* of *A. thaliana* (*A.t.*; Magnotta and Gogarten, 1998; acc. no. O23654, unpublished), and *Brassica napus* (*B.n.*, acc. no. Q39291 [41]), 7 could be assigned to *A*-subunit fragments of *Beta vulgaris* (*B.v.*; Kirsch, 1996; acc. no. Q39442, unpublished), *Daucus carota* (*D.c.*, acc. no. P09469 [42]), *Vigna radiata* (*V.r.*, acc. no. P13548 [43]) and *Gossypium hirsutum* (*G.h.*, acc. no. P31405 [44]). A lower number of cleavage products could be assigned to subunit *A* of *Zea mays* (*Z.m.*; Perotti et al., 1995; acc. no. P49087, unpublished), *H. vulgare* (*H.v.*; DuPont and Chan, 1995; acc. no. Q40002, unpublished), *Acer pseudoplatanus* (*A.p.*; Fraichard et al. 1995, acc. no. U36438, unpublished) and *Allium cepa* (*A.c.*; Pither-Joyce 1999, acc. no. AAF18994, unpublished). In part, this can be explained by the fact that only partial subunit *A* amino acid sequences of the latter four plant species are known.

The MALDI spectrum of the total tryptic proteolysis assay of *N.t.*-53 showed a variety of cleavage products. According to these signals *N.t.*-53 could be identified as subunit *B* of the tobacco V-ATPase. A data base search revealed that the protonated masses of 13 cleavage products (*m*/*z* 1034.56, 1104.51, 1127.54, 1229.58, 1255.64, 1261.60, 1579.92, 1596.95, 1689.98, 1715.98, 2050.98, 2487.14, 2772.39) matched the *m*/*z* values of theoretically produced tryptic fragments of the tobacco subunit *B* sequence deduced from the DNA-sequence determined by Pandey et al. (2000, acc. no. AF220611, unpublished). The detected cleavage products cover 28% of the amino acid sequence (Fig. 2). Two cleavage products only appeared in their modified form including one (*m*/*z* 1104.51) or two (*m*/*z* 2487.14) oxidized methionine residues. The cleavage product *m*/*z* 1579.92 is a modification of fragment *m*/*z* 1596.95 (amino acids 365–378), with a pyroglutamine resulting from cyclization of the N-terminal glutamine residue and removal of an ammonia (17 Da).

It was not possible to obtain a suitable MALDI spectrum for *N.t.*-50. The reason for this might be that the protein amount of this polypeptide was too low. Thus, unfortunately, the only evidence that *N.t.*-50 might represent subunit *H* comes from apparent molecular mass comparison with subunits *H* of yeast and animal sources [45,46].

Two polypeptides were detected in the 40 kDa range, i.e. *N.t.*-41 and *N.t.*-40. A data base search for the tryptic fragments obtained from *N.t.*-41 led to the unequivocal identification of *N.t.*-41 as subunit *C*. Although the tobacco subunit *C* gene is not identified yet and subunits *C* from different organisms show essential sequence differences [47], four of the detected cleavage products (see Table 2) matched *m*/*z* values of fragments of the theoretically pro-

Table 1
Protonated molecular masses of tryptic fragments of the polypeptide *N.t.*-64 in comparison to data base information on V–ATPase *A*-subunits of different plant species (indicated by their data base accession numbers)

N.t.-64	Protonated molecular mass [Da]	Amino acid position	Amino acid sequence (according to *A.t.* O23654)	*A.t.* O 23654	*B.n.* Q 39291	*B.v.* Q 39442	*D.c.* P 09469	*V.r.* P 13548	*G.h.* P 31405	*Z.m.* P 49087	*H.v.* Q 40002	*A.p.* U 36438	*A.c.* AAF 18994
1	1255.69	223–234	(K) LAADTPLLTGQR (V)	*	*	*	*	*	*	*	*	*	*
2	1335.73	48–59	(R) VGHDNLIGEIIR (L)	*	*	*	*		*	n.d.		n.d.	n.d.
3	1545.77[a]	312–326	(R) TTLVANTSNMPVAAR (E)	*	*	*	*	*	*	*	*	*	*
4	1733.89	327–341	(R) EASIYTGITIAEYFR (D)	*	*	*	*	*	*	*	*	*	*
5	1801.94	546–560	(R) NIIHFYNLANQAVER (G)	*	*	*	*	*				n.d.	n.d.
6	1795.86[a]	368–384	(R) LAEMPADSGYPAYLAAR (L)	*	*	*	*	*	*	*		*	*
7	2097.12	484–501	(R) EVLQREDDLNEIVQLVGK (D)	*	*	*	*	*	*	*	*	n.d.	n.d.
8	2220.06[a]	26–47	(R) KVSGPVVVADGMAGAAMYELVR (V)	*	*			*	*	n.d.	n.d.	n.d.	n.d.

The positions of amino acids and their sequence are given according to the V-ATPase *A*-subunit of *A. thaliana* O23654. Amino acids in brackets: sites of tryptic degradation. Asterisks: identical *m*/*z*-ratios at a mass precision of 0.1 Da.

n.d. = not detected, since this fragment is not present in the known partial amino acid sequences of *Z.m.*, *H.v.*, *A.p.* and *A.c.*

[a] Degradation product was found in the MALDI-MS spectrum only in its modified form, i.e. with one or two oxidized methionine residues, i.e. exhibiting a protonated molecular mass increased by *m*/*z* 16 or *m*/*z* 32.

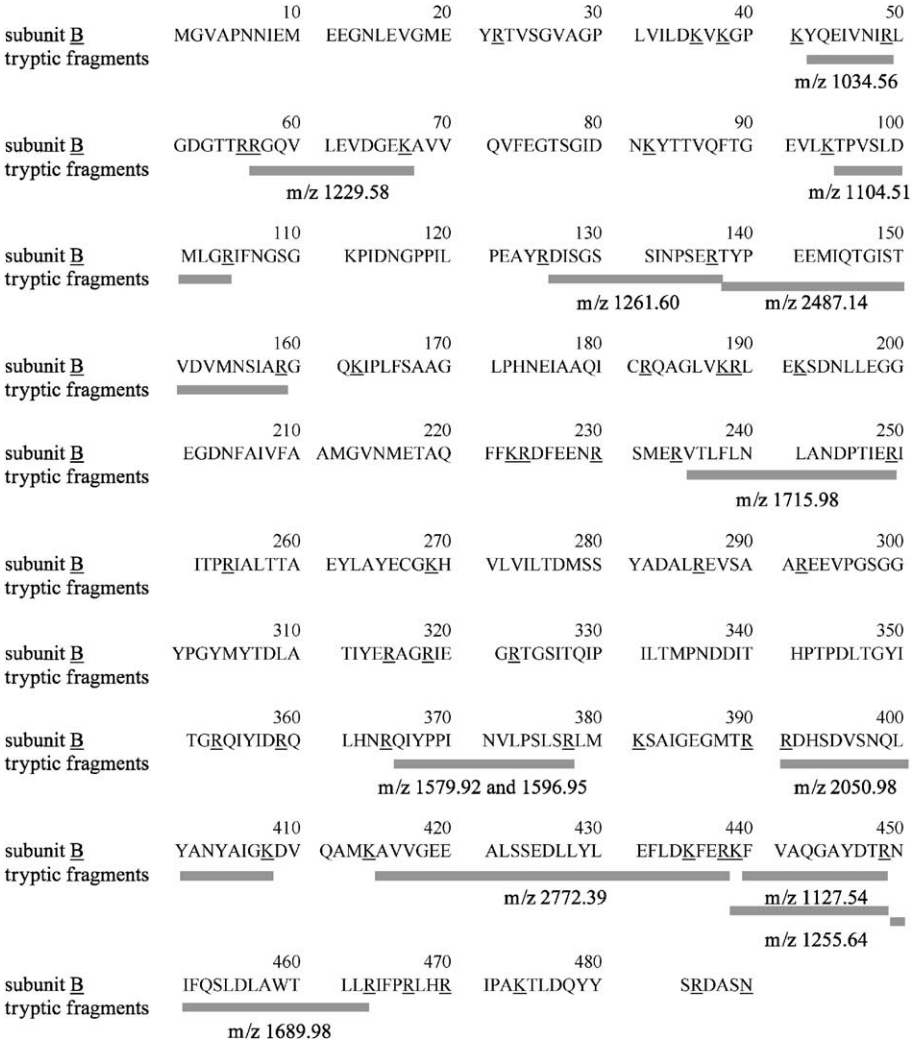

Fig. 2. Comparison of the tryptic fragment of the polypeptide *N.t.*-53 (bars) detected by MALDI-MS with the amino acid sequence of the tobacco V-ATPase subunit *B* (Pandey et al., 2000; acc. no. AF220611, unpublished). Underlined characters indicate potential trypsin cleavage sites.

teolyzed *A. thaliana* subunit *C* (acc. no. AAF20146 [48]). MALDI-PSD analysis of the cleavage product *m/z* 873.48 supported this result since 9 out of 10 *m/z* values of the obtained fragment ions matched. Another tryptic fragment (*m/z* 1135.52) of *N.t.*-41 was shown to be present in the *H. vulgare* subunit *C* (acc. no. AJ242873; amino acids 35–43

Table 2
Protonated molecular masses of tryptic fragments of the polypeptide *N.t.*-41 compared with data base information on V-ATPase subunit-*C* and EST-clones of different plant species as identified by their accession numbers

N.t.-41	Protonated molecular mass [Da]	Amino acid position	Amino acid sequence (After *A.t.* AAF20146 unless otherwise stated)	*A.t.* AAF 20146	*H.v.* AJ 242873	*L.e.* AW 218960	*G.m.* AI 442245	*G.h.* AI 727368	*O.s.* D 24151
1	873.48	39–45	(R) FNIPNLR (V)	*		*	*	*	
2	916.47	76–82	(R) QIEELER (I)	*		*	*	*	*
3	936.47[a]	112–119	(K) YPTMSPLK (E)	*		*	n.d.		n.d.
4	1072.55	75–82	(R) RQIEELER (I)	*		*	*	*	*
5	1135.52	35–43	(R) HSFDTPVYR (F) (R) HSFDTPLYR (F) after *H.v.* AJ242873		*	*	*	*	*

As far as not mentioned otherwise all amino acid positions and sequences are given according to the *A.t.* subunit *C* (AAF20146). Amino acids in brackets indicate tryptic cleavage sites. Asterisks indicate identical *m/z* values at a molecular mass precision of 0.1 Da.
n.d. = not detected, since this fragment is not present in the EST clones.

[a] The cleavage product corresponding to amino acid position 112–119 was only detected in the MALDI-MS spectrum in its modified form containing an oxidized methionine residue, i.e. exhibiting a molecular mass which was 16 Da larger than expected.

[47]). In the corresponding amino acid sequence of the *A. thaliana* subunit *C* (amino acids 30–38) a lysine residue is exchanged by a valine residue resulting in a lower molecular mass. Taking this into account the cleavage product *m*/*z* 1135.52 would also fit into the *A. thaliana* subunit *C* sequence. On the basis of the *A. thaliana* and *H. vulgare* subunit *C* sequences EST data base searches were performed. These searches showed matches of *m*/*z* values of *N.t.*-41 fragments with respective theoretical fragments of the deduced amino acid sequences of EST clones from *Lycopersicon esculentum* (*L.e.*; van der Hoeven et al. 1999, acc. no. AW218960, unpublished), *Glycine max* (*G.m.*; Shoemaker et al. 1999, acc. no. AI442245, unpublished), *G. hirsutum* (*G.h.*; Blewitt et al. 1999, acc. no. AI727368, unpublished) and *Oryza sativa* (*O.s.*; Minobe and Sasaki 1995, acc. no. D24151, unpublished), which putatively are fragments of subunit *C* genes (see Table 2).

It was not possible to identify the polypeptide *N.t.*-40 by a data base search using the *m*/*z* values of the tryptic fragments resolved by MALDI-MS. Thus, the fragment exhibiting the most pronounced signal peak (*m*/*z* 1076.49; data not shown) was selected and its fragment ion spectrum was determined by MALDI-PSD analysis. Using the protonated masses of the fragment ions a data base search led to the identification of an eight-amino-acid long sequence present in EST clones of *A. thaliana* (*A.t.*), *L. esculentum* (*L.e.*; flower: van der Hoeven et al., 1999, acc. no. AW218033, unpublished; fruit: Alcala et al., 1999, acc. no. AW223386, unpublished), *Lotus japonicus* (*L.j.*; Poulsen and Poedenphant, 1999, acc. no. AW164045, unpublished) and *G. max* (*G.m.*; Shoemaker et al., 1999, acc. no. AW233997, unpublished) by identity of 18 of the 21 fragment ions detected. The amino acid sequences deduced from the *L. japonicus* and *G. max* EST sequences exhibited similarities to the V-ATPase subunit AC39 (accessory V-ATPase subunit), which is also known as subunit *d*. The amino acid sequence of the cleavage product *m*/*z* 1076.49 showed 88% identity to the respective partial amino acid sequence of the *S. cerevisiae* (acc. no. P32366 [49]) and *Dictyostelium discoideum* (acc. no. P54641 [50]) subunit *d*, and 75% identity to the *Bos taurus* (acc. no. P12953 [51]) and *Homo sapiens* (acc. no. Q02547 [52]) subunit *d*. Thus, for the first time subunit *d*, which is suggested to be part of the V_o domain, could be identified unequivocally to be a constituent of the *N. tabacum* V-ATPase holoenzyme. After theoretical tryptic proteolysis of the 200-amino-acid long polypeptide deduced from the *L. esculentum* EST clone (flower, acc. no. AW218033, van der Hoeven et al., 1999, unpublished), in addition to the cleavage product *m*/*z* 1076.49, four additional cleavage products of *N.t.*-40 could be identified (Table 3). Moreover, the molecular mass of several *N.t.*-40 cleavage products matches theoretical tryptic fragments of polypeptides from two isoforms of *A. thaliana* subunit *d* (acc. no. Q9LHA4 and Q9LJ5; [12]) and from EST clones of other plant species.

Four polypeptides have been detected in the apparent molecular mass range around 30 kDa, i.e. *N.t.*-31, *N.t.*-30, *N.t.*-29 and *N.t.*-28. These polypeptides were candidates for subunits *D* and *E*. However, it remained absolutely open which of these polypeptides could be assigned to the respective subunits and why four instead of the expected two polypeptides were present in the fraction of immunoprecipitated V-ATPase. Interestingly, only three of these four polypeptides were labeled by an antiserum directed against the *Kalanchoë daigremontiana* V-ATPase holoenzyme in a previous study [19], making the situation even more confusing.

A data base search using the molecular masses of tryptic fragments resolved by MALDI analysis led to the unequivocal identification of the polypeptide *N.t.*-30 as subunit *D*. Six tryptic fragments (*m*/*z* 713.29, 859.40, 960.39, 1036.50, 1164.60, 1654.77) matched theoretical tryptic fragments of subunit *D* of *A. thaliana* (acc. no. CAB46439 [53]). The tryptic fragments covered 11% of the total *A. thaliana* subunit *D* sequence (amino acid positions 37–46, 121–128, 191–203). In parallel to MALDI analysis the N-terminal amino acid sequence of one of the tryptic fragments was determined after purification by capillary electrophoresis as EDFLENVVKPK. This sequence showed a similarity of 73% and 64% with the respective sequences of the

Table 3
Protonated molecular masses of tryptic fragments of the polypeptide *N.t.*-40 compared with data base information on EST-clones of different plant species and two subunit-*d* isoforms of *A. thaliana* as identified by their accession numbers

N.t.-40	Protonated molecular mass [Da]	Amino acid position	Amino acid sequence (after *L.e.* AW218033)	*L.e.* AW 218033	*L.e.* AW 223386	*L.j.* AW 164045	*G.m.* AW 233997	*A.t.* Q 9LHA4	*A.t.* Q 9LJI5
1	1076.49	125–132	(K) AYLEDFYR (F)	*	*	*	*		
2	1601.95	157–171	(R) AVNITINSIGTELTR (D)	*	n.d.		*	*	*
3	1995.93	137–155	(K) LGGATAEIMSDLLSFEADR (R)	*	n.d.				
4	2152.04	137–156	(K) LGGATAEIMSDLLSFEADRR (A)	*	n.d.				
5	2167.09[a]	39–57	(R) YGHMIDNVVLIVTGTLHER (D)	*	*	n.d.	n.d.	*	*

Amino acid positions and sequences are given according to the polypeptide deduced from the *L. esculentum* AW218033 EST clone. Amino acids in brackets indicate tryptic cleavage sites. Asterisks indicate identical *m*/*z* values at a molecular mass precision of 0.1 Da.
n.d. = not detected, since this fragment is not present in the EST clones.
[a] The cleavage product corresponding to amino acid position 39–57 was only detected in the MALDI-MS spectrum in its modified form containing an oxidized methionine residue, i.e. exhibiting a molecular mass which was 16 Da larger than expected.

Table 4
Comparison of the N-terminal sequence of a tryptic fragment of the polypeptide *N.t.*-30 with the respective sequence of subunits *D* of other organisms identified by their data base accession number

N-terminal amino acid sequence of a tryptic fragment of *N.t.*-30	E	D	F	L	E	N	V	V	K	P	K
Arabidopsis thaliana (CAB46439)[a]	V	N	A	*	*	*	*	*	*	*	*
Pisum sativum [b]	V	N	A	*	*	*	*	*	*	*	R
Bos taurus (P39942)[c]	V	N	R	I	*	H	*	I	I	*	R
Oryctolagus cuniculus (AAD10366)[d]	V	N	A	I	*	H	*	I	I	*	R
Caenorhabditis elegans (P34462)[e]	V	N	A	I	*	H	*	I	I	*	R
Candida albicans (P87220)[f]	V	N	A	I	*	H	*	I	I	*	R
Saccharomyces cerevisiae (P32610)[g]	V	N	A	I	*	H	*	I	I	*	R
Neurospora crassa (O59941)[h]	V	N	A	I	*	H	*	I	I	*	R
Thermus thermophilus (BAA33198)[i]	V	N	A	*	*	Q	*	*	I	*	G
Methanococcus jannaschii (Q58032)[j]	V	N	A	*	*	Y	*	I	I	*	R
Methanobacterium thermoautotrophicum (AAB85449)[k]	V	N	A	*	*	H	I	I	I	*	R

Asterisks indicate identical amino acids.
[a] [53].
[b] [64].
[c] [65].
[d] [66].
[e] [67].
[f] Janbon et al. (1997) unpublished.
[g] Mulligan et al. (1993) unpublished.
[h] Margolles-Clarke and Bowman (1998) unpublished.
[i] Yokoyama (1995) unpublished.
[j] [68].
[k] [69].

A. thaliana and *Pisum sativum* subunit *D* sequences, respectively, and lower similarities with the subunit *D* sequences known from other organisms (Table 4).

The polypeptides *N.t.*-31, *N.t.*-29 and *N.t.*-28 were identified as different forms of subunit *E* by MALDI analysis. Tables 5–7 show the comparison of tryptic fragments of *N.t.*-31, *N.t.*-29 and *N.t.*-28, respectively, with theoretical tryptic fragments of subunits *E* of *C. limon* (*C.l.*; Reuveni and Sadka, 1999, acc. no. AAD49706, unpublished), *H. vulgare* (*H.v.*; Ford et al., 1997, acc. no. AAD10336, unpublished), *Z. mays* (*Z.m.*; Stapleton and Walbot 1999, acc. no. AAD45282, unpublished), *M. crystallinum* (*M.c.*, acc. no. Q40272 [54]), *G. hirsutum* (*G.h.*, acc. no. AAB72177 [55]), *Spinacia oleracea* (*S.o.*, acc. no. CAA 65581 [56]) and *A. thaliana* (*A.t.*; Bevan et al., 1999, acc. no. CAB43050, unpublished; *A.t.*; acc. no. Q39258 [56]). Since the amino acid positions are given according to the *C. limon* subunit *E* sequence the amino acid position of the tryptic cleavage sites can vary between species, although the amino acid sequence of the cleavage product

Table 5
Protonated molecular masses of tryptic fragments of the polypeptide *N.t.*-31 compared with data base information on subunits *E* of different plant species as identified by their accession numbers

N.t.-31	Protonated molecular mass [Da]	Amino acid position	Amino acid sequence (after *C.l.* AAD49706 unless otherwise stated)	*C.l.* AAD 49706	*H.v.* AAD 10336	*Z.m.* AAD 45282	*G.h.* AAB 72177	*M.c.* Q 40272	*S.o.* CAA 65581	*A.t.* CAB 43050	*A.t.* Q 39258
1	755.46	216–221	(K) KLPEIR (K)	*	*	n.d.	*[a]				
		217–222	(K) LPEIRK (Q)	*	*	n.d.	*[a]				
2	762.44	209–214	(R) LDVVFR (K)			n.d					
		209–214	(R) LEVVFR (K) after *H.v.* AAD10336		*	n.d.					
3	902.44[b]	9–15	(K) QIQQMVR (F)	*	*	*	*		*	*	*
4	1054.63	125–133	(R) LKEPSVLLR (C) after *A.t.* CAB43050			n.d.				*	*
5	1311.61[b]	68–78	(K) IEYSMQLNASR (I)	*	*	*	*	*	*		
6	1808.86	25–40	(K) ANEISVSAEEEFNIEK (L)	*		*	*	*		*	

Amino acid positions and sequences are given according to the polypeptide deduced from the *C. limon E*-subunit AAD49706. Amino acids in brackets indicate tryptic cleavage sites. Asterisks indicate identical *m/z* values at a molecular mass precision of 0.1 Da.
n.d. = not detected, since this fragment is not present in the EST clone of *Z.m.*
[a] In *G.h.* this corresponds to amino acid positions 223–228 and 224–229.
[b] The cleavage product was only detected in the MALDI-MS spectrum in its modified form containing an oxidized methionine residue, i.e. exhibiting a molecular mass which was 16 Da larger than expected.

Table 6
Protonated molecular masses of tryptic fragments of the polypeptide *N.t.*-29 compared with data base information on subunits *E* of different plant species as identified by their accession numbers

N.t.-29	Protonated molecular mass [Da]	Amino acid position	Amino acid sequence (after *C.l.* AAD49706 unless otherwise stated)	*C.l.* AAD 49706	*H.v.* AAD 10336	*Z.m.* AAD 45282	*G.h.* AAB 72177	*M.c.* Q 40272	*S.o.* CAA 65581	*A.t.* CAB 43050	*A.t.* Q 39258
1	724.31	53–57	(R) QEYER (K)	*	*	*		*			
2	755.46	216–221	(K) KLPEIR (K)	*	*	n.d.	*[a]				
		217–222	(K) LPEIRK (Q)	*	*	n.d.	*[a]				
3	762.44	209–214	(R) LDVVFR (K)								
		209–214	(R) LEVVFR (K) after *H.v.* AAD10336		*	n.d.					
4	902.44[b]	9–15	(K) QIQQMVR (F)	*	*	*	*		*	*	*
5	1054.63	125–133	(R) LKEPSVLLR (C) after *A.t.* CAB43050			n.d.				*	*
6	1311.61[b]	68–78	(K) IEYSMQLNASR (I)	*	*	*	*	*	*		
7	1808.86	25–40	(K) ANEISVSAEEEFNIEK (L)	*		*	*	*		*	

Amino acid positions and sequences are given according to the polypeptide deduced from the *C. limon E*-subunit AAD49706. Amino acids in brackets indicate tryptic cleavage sites. Asterisks indicate identical *m*/*z* values at a molecular mass precision of 0.1 Da.
n.d. = not detected, since this fragment is not present in the EST clone of *Z.m.*
[a] In *G.h.* this corresponds to amino acid positions 223–228 and 224–229.
[b] The cleavage product was only detected in the MALDI-MS spectrum in its modified form containing an oxidized methionine residue, i.e. exhibiting a molecular mass which was 16 Da larger than expected.

is identical. Up to now, for the *Z. mays* subunit *E* only a partial sequence consisting of 128 amino acids is published. Thus, not all hypothetical cleavage products can be compared in this case. Since it could not be clarified if the cleavage product *m*/*z* 755.46 corresponds to amino acid positions 216–221 or 217–222, both sequences were included in the tables. Interestingly, for the polypeptides *N.t.*-31, *N.t.*-29 and *N.t.*-28 different numbers of tryptic fragments were detected, which could be assigned to theoretical cleavage products of subunits *E* of different species. The highest number of matches, indicating a high degree of sequence identity, was found for the *C. limon* and the *H. vulgare* subunit *E*. Subunits *E* from other species showed a lower number of matches (see Tables 5–7). It has to be mentioned that differences in amino acid sequences cannot unequivocally be determined by MALDI analysis, however, it is possible to obtain hints about the sequence identity of two polypeptides. In this respect, the comparison of protonated molecular masses of cleavage products indicates a high degree of sequence identity between the *N.t.*-31 and *N.t.*-29, while *N.t.*-28 exhibits distinct differences compared to the other two polypeptides. As an example, the cleavage product *m*/*z* 1054.63 was not detected in the case of *N.t.*-28, while the cleavage products *m*/*z* 1410.86

Table 7
Protonated molecular masses of tryptic fragments of the polypeptide *N.t.*-28 compared with data base information on subunits *E* of different plant species as identified by their accession numbers

N.t.-28	Protonated molecular mass [Da]	Amino acid position	Amino acid sequence (after *C.l.* AAD49706 unless otherwise stated)	*C.l.* AAD 49706	*H.v.* AAD 10336	*Z.m.* AAD 45282	*G.h.* AAB 72177	*M.c.* Q 40272	*S.o.* CAA 65581	*A.t.* CAB 43050	*A.t.* Q 39258
1	724.31	53–57	(R) QEYER (K)	*	*	*		*			
2	755.46	216–221	(K) KLPEIR (K)	*	*	n.d.	*[a]				
		217–222	(K) LPEIRK (Q)	*	*	n.d.	*[a]				
3	762.44	209–214	(R) LDVVFR (K)								
		209–214	(R) LEVVFR (K) after *H.v.* AAD10336		*	n.d.					
4	902.44[b]	9–15	(K) QIQQMVR (F)	*	*	*	*		*	*	*
5	993.48	51–57	(K) IRQEYER (K)	*	*	*		*			
6	1311.61[b]	68–78	(K) IEYSMQLNASR (I)	*	*	*	*	*	*		
7	1410.86	113–124	(K) LLKDLIVQSLLR (L)	*							
8	1808.86	25–40	(K) ANEISVSAEEEFNIEK (L)	*		*	*	*		*	
	1818.92	25–40	(K) ANEISVPAEEEFNIEK (L) after *A.t.* Q39258								*

Amino acid positions and sequences are given according to the polypeptide deduced from the *C. limon E*-subunit AAD49706. Amino acids in brackets indicate tryptic cleavage sites. Asterisks indicate identical *m*/*z* values at a molecular mass precision of 0.1 Da.
n.d. = not detected, since this fragment is not present in the EST clone of *Z.m.*
[a] In *G.h.* this corresponds to amino acid positions 223–228 and 224–229.
[b] The cleavage product was only detected in the MALDI-MS spectrum in its modified form containing an oxidized methionine residue, i.e. exhibiting a molecular mass which was 16 Da larger than expected.

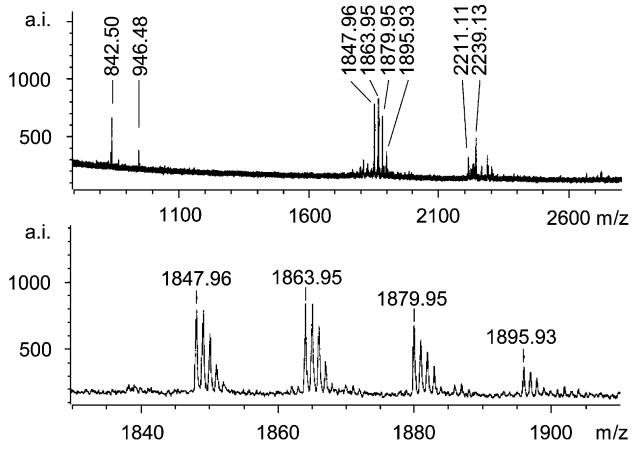

Fig. 3. Positive ion MALDI mass spectrum of the total tryptic digest of the *N.t.*-16 polypeptide within the detection window of *m/z* 700–2800 (top panel) and an expanded part of the total spectrum showing the group of four tryptic peptides exhibiting *m/z* values in the range of 1847.96–1895.93 Da (bottom panel). a.i.: arbitrary intensity.

and *m/z* 1818.92 exclusively were detected for *N.t.*-28. Moreover, the differential occurrence of the cleavage products *m/z* 724.31 and *m/z* 993.48 indicates differences in the amino acid sequences of *N.t.*-31, *N.t.*-29 and *N.t.*-28. For *N.t.*-28 both cleavage products could be detected. While the lack of the two cleavage products in *N.t.*-31 might be due to a lower protein amount compared to *N.t.*-29 and *N.t.*-28, the lack of *m/z* 993.48 indicates a difference in amino acid sequence in the region of the tryptic cleavage site. Thus, MALDI analysis clearly indicates the occurrence of three distinct subunit *E* isoforms in tobacco.

Only a single cleavage product (*m/z* 1847.96) was detected within the detection window of *m/z* 700–2800 in the MALDI spectrum (Fig. 3) of the tryptic digest of the *N.t.*-16 polypeptide. This cleavage product was part of a group of four signals (*m/z* 1847.96–1895.93). All other signals were trypsin autolysis products or well-known artifact signals typically occurring in MALDI spectra of proteolytically cleaved polypeptides. The difference in molecular mass between the cleavage products appearing of the *m/z* 1847.96–1895.93 group was 16 Da each. This can be explained by the fact that they result from a single cleavage product containing three methionine residues. While *m/z* 1847.96 does not contain any oxidized methionine, *m/z* 1863.95, *m/z* 1879.95 and *m/z* 1895.93 contained one, two and three oxidized methionine residues, respectively. Using an ion selector, it was possible to record separate spectra for each of the products (data not shown). In these spectra the occurrence of additional signals was observed exhibiting molecular masses lower by 64 Da, caused by loss of methane sulfenic acid due to metastable fragmentation. A similar signal pattern was previously observed during analysis of synthetic peptides and shown to arise from oxidized methionine residues [57,58]. By theoretical tryptic proteolysis of the tobacco subunit *c* sequence a cleavage product (amino acid residues 37–54) could be identified exhibiting an *m/z* value of 1847.96 and containing three methionine residues. Thus, *N.t.*-16 could be assigned to the proton channel subunit *c* of the tobacco V-ATPase.

It was not possible to identify the polypeptide *N.t.*-15 according to the MALDI spectrum of its tryptic digest although several cleavage products were detected. Thus, the most pronounced cleavage product (*m/z* 1608.73) was chosen for MALDI-PSD analysis. A data base search of protonated molecular masses of 18 fragments revealed the identity of 15 fragments with respective amino acid sequences of the *A. thaliana* subunit *F* ([12], acc. no. Q9ZQX4). The amino acid sequence similarity of the respective sequence region of subunits *F* of *H. sapiens* (acc. no. Q16864 [59]) or *B. taurus* (acc. no. Q28029 [60]) was identical, while subunit *F* of *S. cerevisiae* (acc. no. P39111 [61]) showed a sequence similarity of 79%. Thus, according to the results for *N.t.*-15, for the first time subunit *F* could be detected as a constituent of the tobacco V-ATPase.

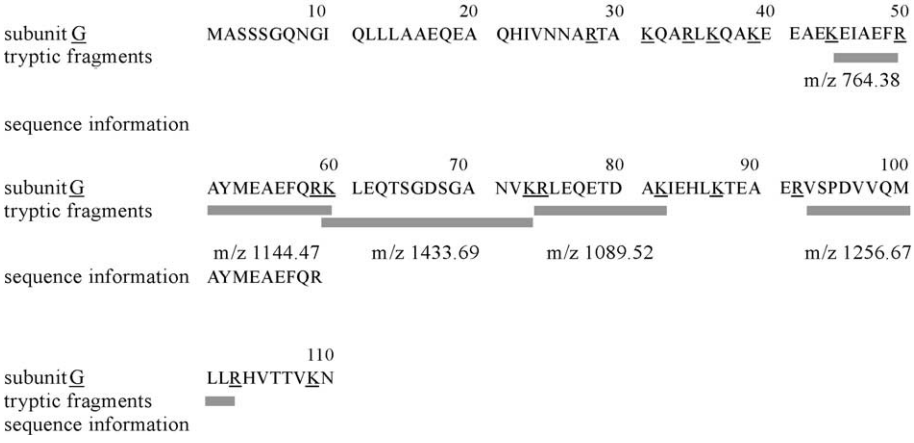

Fig. 4. Comparison of the tryptic fragments of the polypeptide *N.t.*-13 (bars) detected by MALDI-MS with the amino acid sequence of the tobacco V-ATPase subunit *G* (acc. no. AJ005899, Nt-vag 1 protein [21]) and position of the amino acid sequence of the cleavage product *m/z* 1244.47. Underlined characters indicate potential trypsin cleavage sites.

Moreover, data base searches using the *N.t.*-15 cleavage product *m*/*z* 1608.73 showed 100% of sequence similarity with deduced amino acid sequences from EST clones of *L. esculentum* (Ganal et al., 1998, acc. no. AA824973, unpublished), *G. max* (Shoemaker et al., 1999, acc. no. AI736385, unpublished), *G. hirsutum* (Blewitt et al., 1999, acc. no. AI725681, unpublished) and *O. sativa* (Sasaki and Minobe, 1994, acc. no. C98270, unpublished), indicating that these plant species also contain subunit *F*.

By a data base search *N.t.*-13 could be unequivocally identified as subunit *G*. Five of the detected cleavage products (*m*/*z* 764.38; 1089.52; 1144.47; 1256.67; 1433.69) perfectly matched the *m*/*z* values of theoretical cleavage products of the tobacco Nt-vag 1 subunit *G* protein isoform ([21], acc. no. AJ005899). The cleavage products covered 45% of the Nt-vag 1 protein sequence (Fig. 4). Moreover, the N-terminal amino acid sequence of the cleavage product *m*/*z* 1144.47 was determined as AYMEAEFQR, perfectly matching the amino acid sequence 51–59 deduced from the Nt-vag 1 gene. Also the second tobacco subunit *G* isoform, the Nt-vag 2 protein ([21], acc. no. AJ005900) contains this amino acid sequence (positions 52–60). However, in contrast to the Nt-vag 1 protein, only two of the *N.t.*-13 cleavage products resolved by MALDI analysis (*m*/*z* 764.38 and 1144.47) matched the Nt-vag 2 protein amino acid sequence covering only 13% of the total Nt-vag 2 protein amino acid sequence. Thus, as far as for the Nt-vag2 protein, no special tryptic fragment was detected; one can suspect that only the Nt-vag 1 subunit *G* isoform is expressed in tobacco leaf cells.

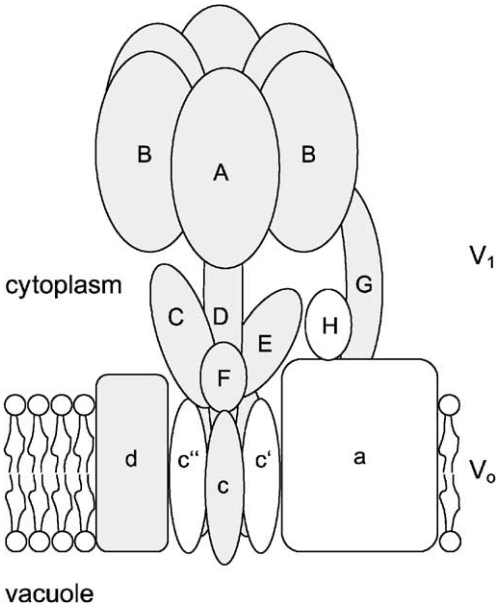

Fig. 5. Working model of the subunit arrangement of the V-ATPase holoenzyme based on biochemical and electron microscopical data as described in Refs. [2,8]. Subunits given in grey have been identified as constituents of the tobacco V-ATPase holoenzyme.

4. Discussion

In fraction of immuno-purified V-ATPase, 11 out of 12 polypeptides could be unequivocally identified as subunits of the tobacco V-ATPase holoenzyme by amino acid sequencing and MALDI-MS analysis. These polypeptides represent subunits *A* (*N.t.*-64), *B* (*N.t.*-53), *C* (*N.t.*-41), *D* (*N.t.*-30), *E* (*N.t.*-31, *N.t.*-29, *N.t.*-28), *F* (*N.t.*-15), *G* (*N.t.*-13), *c* (*N.t.*-16) and *d* (*N.t.*-40). The putative location of these subunits is shown in a working model in Fig. 5.

Unfortunately, polypeptide number 12, i.e. *N.t.*-50, could not be identified, possibly due to a too low protein amount. According to its apparent molecular mass, *N.t.*-50 might represent subunit *H*. However, as already mentioned in Introduction and as demonstrated by the results presented here, such assignment of polypeptides to V-ATPase subunits is insufficient. The apparent molecular mass of a polypeptide is no absolute measure of its real molecular mass. This is mainly caused by incomplete destruction of the tertiary protein structure by the detergent SDS due to strong interaction of respective amino acid residues or by a high amount of negatively charged amino acid residues, hindering the attachment of SDS to the polypeptide and leading to a higher apparent molecular mass value than expected. Moreover, removal of a tightly bound enzyme co-factor or chemical modification by phosphorylation and/or glycosylation may lead to variations in apparent molecular mass [62]. Since respective V-ATPase subunits of different organisms vary in their amino acid composition, it is not astonishing that broad apparent molecular mass ranges have been reported for these subunits (for review see Ref. [2]). The fact that different molecular masses have been reported for a respective V-ATPase subunit of one single plant species might be due to the use of different gel systems or electrophoresis conditions by different investigators. Some examples should underline the insufficiency of the identification of V-ATPase subunits by apparent molecular mass comparison. In the present study, it would have been impossible to decide whether *N.t.*-41 or *N.t.*-40 represents subunit *C* or which of the polypeptides *N.t.*-31, *N.t.*-30, *N.t.*-29 and *N.t.*-28 represent subunit *D* or *E*. By simple apparent molecular mass comparison, *N.t.*-31 would have been identified as subunit *D*, however, MALDI analysis clearly showed that *N.t.*-31 is a subunit *E* isoform.

Four of the subunits, i.e. subunits *a*, *c*′, *c*″ and *H*, suggested to be part of the yeast V-ATPase [11], could not be unequivocally identified to be present in the tobacco V-ATPase. The occurrence of subunit *a* could not be investigated since in the immunoprecipitate too many heavily stained polypeptides exhibiting a molecular mass range around 100 kDa were present, which were also detected in the *anti-A*/protein A-sepharose control (compare Fig. 1, lanes 3 and 4). In the tonoplast vesicle fraction, which was used for V-ATPase purification, several bands exhibiting an apparent molecular mass around 100 kDa

were visible (see Fig. 1, lane 2), which might be candidates for subunit *a*. Thus, it cannot be excluded that this subunit is present in the tobacco V-ATPase holoenzyme, however, we do not have biochemical data to support this suggestion so far. On the other hand, no interfering polypeptide bands derived from the antibody/protein A-sepharose complex were visible in the molecular mass range from 15 to 20 kDa. This is the molecular mass region where subunits *c*′ and *c*″ should be present. Since in addition to *N.t.*-16 and *N.t.*-15, which were identified as subunit *c* and subunit *F*, respectively, no other polypeptides were detected in this molecular mass region, it can be suggested that subunit *c*′ and *c*″ are not present in the tobacco V-ATPase holoenzyme. One might suspect that these subunits were lost during solubilization of tonoplast proteins prior to immunoprecipitation. However, in view of the structural similarity of subunits *c*, *c*′ and *c*″ [11], which all are highly hydrophobic integral membrane proteins, the probability is low that subunits *c*′ and *c*″ selectively were removed, while subunit *c* was still present in the immuno-precipitated V-ATPase. Moreover, Triton X-100 is a relatively mild non-ionic detergent. In previous experiments solubilization of tonoplast proteins of *M. crystallinum* or *K. daigremontiana* [63] at 1% (w/v) and 2% (w/v) Triton X-100, respectively, with subsequent chromatographical purification led to fractions of active V-ATPase, indicating that all subunits necessary for activity were present in the purified V-ATPase holoenzyme after Triton X-100 treatment. Another interesting point with respect to a possible loss of subunits during solubilization and immuno-purification is the fact that the protein amount of the *N.t.*-50 polypeptide was too low for a successful MALDI analysis. Since only V-ATPase subunits were found in the immuno-precipitate, while other tonoplast proteins were not present, immunoprecipitation seems to be an effective and selective method for V-ATPase purification. Thus, it is reasonable to assume that also *N.t.*-50 represents a V-ATPase subunit, i.e. subunit *H*. If this is true, the question arises why all other V-ATPase subunits were present in sufficient amounts in the immuno-precipitate while *N.t.*-50 was not. According to Wilkens et al. [8], all accessory V-ATPase subunits, with the exception of subunit *H*, are required for a structural and functional assembly of V_1, indicating a somewhat peripheral location of this subunit. Thus, it might be suspected that subunit *H* compared to other subunits is more loosely bound to the holoenzyme and that this subunit in part was removed during purification.

In tobacco genes for at least two isoforms of subunits *G* ([21]; Nt-vag 1 and Nt-vag 2) and *c* (Kirsch, 1996, unpublished; acc. nos. Q40585 and Q40560) have been cloned. However, there was no evidence from MALDI analysis that different isoforms of both subunits were present in the tobacco leaf V-ATPase. The different subunit isoforms might be expressed in different types of tissue or under special environmental or metabolic conditions. In the case of subunit *c*, there is evidence that different isoforms are expressed in response to nitrate nutrition of the plant, i.e. growth at 2 and 40 mM nitrate [36]. In additional experiments (data not shown), we investigated *N.t.*-16 from tobacco plants grown at low and high nitrate nutrition, respectively. However, MALDI analysis of *N.t.*-16 from tobacco plants grown at 2 mM nitrate led to the identification of the identical single tryptic fragment obtained as for *N.t.*-16 from tobacco plants grown at 20 mM nitrate. Thus, additional experiments, e.g. cleavage of *N.t.*-16 with other proteases prior to MALDI analysis to obtain a larger amount of proteolytic cleavage products resolvable in the detection window, are necessary to verify whether different mineral supply leads to expression of different subunit *c* isoforms in tobacco.

An important result of the present study is the identification of three subunit *E* isoforms present in the tobacco V-ATPase. Two distinct subunit *E* isoforms previously have been found in *P. sativum* [64]. Interestingly, one isoform (E2) seemed to be a constitutive form present in all tissues investigated, while the other isoform (E1) was only hardly detected in preparations from leaf and cotelydon tissue. In the present study, all three subunit *E* isoforms identified were present in one single tissue, i.e. leaf tissue, leading to the assumption that in the tonoplast of tobacco leaf cells various populations of V-ATPase holoenzymes containing different subunit *E* isoforms are present. This might have physiological significance since in *P. sativum*, it has been shown that the occurrence of different *E*-subunits in the holoenzyme has an impact on the kinetic parameters of ATP-hydrolysis activity [64]. In addition, V-ATPases have been demonstrated to be located in different cellular membranes (for review see Ref. [2]). However, at present, no data are available to assign specific functions to isotypes of the V-ATPase holoenzyme present in the tobacco leaf tonoplast or to V-ATPases present in different endomembranes.

Taken together, nine subunits of the tobacco V-ATPase could be unequivocally identified to be constituents of the tobacco leaf cell V-ATPase holoenzyme. Moreover, three different isoforms of subunit *E* were detected, indicating the occurrence of different populations of V-ATPases within one single tissue. The data obtained at the protein level presented here will facilitate the identification of different subunits of the plant V-ATPase, and open the possibility to assign changes of polypeptides to variations in the occurrence of V-ATPase subunits due to environmental and developmental factors in the model plant tobacco.

Acknowledgements

We are grateful to Dr. Tore Kempf (DKFZ Heidelberg, Germany) for the determination of amino acid sequences of proteolytic fragments. This work was supported by the Deutsche Forschungsgemeinschaft (Bonn, Germany) in the

frame of the Sonderforschungsbereich 199 (projects B2 and B3).

References

[1] U. Lüttge, R. Ratajczak, The physiology, biochemistry and molecular biology of the plant vacuolar ATPase, Adv. Bot. Res. 25 (1997) 253–296.

[2] R. Ratajczak, Structure, function and regulation of the plant vacuolar H^+-translocating ATPase, Biochim. Biophys. Acta 1465 (2000) 17–36.

[3] R. Ratajczak, T.A. Wilkins, Energizing the tonoplast, Annu. Plant Rev. 5 (2000) 133–173.

[4] R. Klink, U. Lüttge, Electron-microscopic demonstration of a "head and stalk" structure of the leaf vacuolar ATPase in *Mesembryanthemum crystallinum* L., Bot. Acta 104 (1991) 122–131.

[5] S. Lee Taiz, L. Taiz, Ultrastructural comparison of the vacuolar and mitochondrial H^+-ATPases of *Daucus carota*, Bot. Acta 104 (1991) 117–121.

[6] W.J. Dschida, B.J. Bowman, Structure of the vacuolar ATPase from *Neurospora crassa* as determined by electron microscopy, J. Biol. Chem. 267 (1992) 18783–18789.

[7] E. Boekema, T. Ubbink-Kok, J.S. Lolkema, A. Brisson, W.N. Konings, Visualization of a peripheral stalk in V-type ATPase: evidence for the stator structure essential to rotational catalysis, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 14291–14293.

[8] S. Wilkens, E. Vasilyeva, M. Forgac, Structure of the vacuolar ATPase by electron microscopy, J. Biol. Chem. 274 (1999) 31804–31810.

[9] W. Junge, H. Lill, S. Engelbrecht, ATP synthase: an electrochemical transducer with rotary mechanics, Trends Biochem. Sci. 22 (1997) 420–423.

[10] M. Grabe, H. Wang, G. Oster, The mechanochemistry of V-ATPase proton pumps, Biophys. J. 78 (2000) 2798–2813.

[11] T.H. Stevens, M. Forgac, Structure, function and regulation of the vacuolar (H^+)-ATPase, Annu. Rev. Cell Dev. Biol. 13 (1997) 779–808.

[12] The *Arabidopsis Initiative*, Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*, Nature 408 (2000) 796–815.

[13] J.M. Ward, H. Sze, Subunit composition and organization of the vacuolar H^+-ATPase from oat roots, Plant Physiol. 99 (1992) 170–179.

[14] F.M. DuPont, P.J. Morrissey, Subunit composition and Ca-ATPase activity of the vacuolar ATPase from barley roots, Arch. Biochem. Biophys. 294 (1992) 341–346.

[15] M. Hosaka, Y. Kanayama, K. Shiratake, S. Yamaki, Tonoplast H^+-ATPase of mature pear fruit, Phytochemistry 36 (1994) 565–567.

[16] R. Verstappen, T. Rausch, Different SH groups involved in H^+ translocation and PP_i hydrolysis of higher plant Mg^{2+}-PP_iase, FEBS Lett. 236 (1988) 420–424.

[17] K.D. Johnson, H. Hoefte, M.J. Crispeels, An intrinsic tonoplast protein of protein storage vacuoles in seeds is structurally related to a bacterial solute transporter (GlpF), Plant Cell 2 (1990) 525–532.

[18] S. Hörtensteiner, E. Martinoia, N. Amrhein, Factors affecting the reformation of vacuoles in evacuolated protoplasts and the expression of the two vacuolar proton pumps, Planta 192 (1994) 395–403.

[19] E. Fischer-Schliebs, E. Ball, E. Berndt, E. Besemfelder-Butz, M.L. Binzel, M. Drobny, D. Mühlenhoff, M.L. Müller, K. Rakowski, R. Ratajczak, Immunological cross-reactions with an antiserum against the V-ATPase of *Kalanchoë daigremontiana* reveal structural differences of V-ATPase subunits of different plants, Biol. Chem. 378 (1997) 1131–1139.

[20] K. Matsuoka, T. Higushi, M. Maeshima, K. Nakamura, A vacuolar-type H^+-ATPase in a nonvacuolar organelle is required for the sorting of soluble vacuolar protein precursors in tobacco cells, Plant Cell 9 (1997) 533–546.

[21] D. Rouquié, C. Tournaire-Roux, W. Szponarski, M. Rossignol, P. Doumas, Cloning of the V-ATPase subunit G in plant: functional expression and sub-cellular localization, FEBS Lett. 437 (1998) 287–292.

[22] M.L. Binzel, P.M. Hasegawa, A.K. Handa, R.A. Bressan, Adaptation of tobacco cells to NaCl, Plant Physiol. 79 (1985) 118–125.

[23] M.L. Narasimhan, M.L. Binzel, E. Perez-Prat, Z. Chen, D.E. Nelson, N.K. Singh, R.A. Bressan, P.M. Hasegawa, NaCl regulation of tonoplast ATPase 70-kilodalton subunit mRNA in tobacco cells, Plant Physiol. 97 (1991) 562–568.

[24] P.-F.L. Chang, B. Damsz, A.K. Kononowicz, M. Reuveni, Z. Chen, Y. Xu, K. Hedges, C.C. Tseng, N.K. Singh, M.L. Binzel, M.L. Narasimhan, P.M. Hasegawa, R.A. Bressan, Alterations in cell membrane structure and expression of a membrane-associated protein after adaptation to osmotic stress, Physiol. Plant. 98 (1996) 505–516.

[25] D. Heineke, K. Wildenberger, U. Sonnewald, L. Willmitzer, H.W. Heldt, Accumulation of hexoses in leaf vacuoles: studies with transgenic tobacco plants expressing yeast-derived invertase in the cytosol, vacuole or apoplasm, Planta 194 (1994) 29–33.

[26] U. Lüttge, T. Pfeifer, E. Fischer-Schliebs, R. Ratajczak, The role of vacuolar malate-transport capacity in crassulacean acid metabolism and nitrate nutrition. Higher malate-transport capacity in ice plant after crassulacean acid metabolism-induction and in tobacco under nitrate nutrition, Plant Physiol. 124 (2000) 1335–1348.

[27] T. Nagata, S. Hasegawa, Events at the border between the M and G1 phases in highly synchronized culture of tobacco BY-2 cells, J. Plant Res. 3 (1993) 29–35.

[28] T. Nagata, F. Kumagai, S. Hasezawa, The origin and organization of cortical microtubules during the transition between M and G1 phases of the cell cycle as observed in highly synchronized cells of tobacco BY-2, Planta 193 (1994) 567–572.

[29] K. Matsuoka, D.C. Bassham, N.V. Raikhel, K. Nakamura, Different sensitivity to Wortmannin of two vacuolar sorting signals indicates the presence of distinct sorting machineries in tobacco cells, J. Cell Biol. 130 (1995) 1307–1318.

[30] J. Kuerpa, D. Herouart, M. Van Montagu, D. Inzé, Differential expression of CuZn-and Fe-superoxide dismutase genes of tobacco during development, oxidative stress and hormonal treatments, Plant Cell Physiol. 38 (1997) 463–470.

[31] E. Tyystjaervi, M. Riikonen, A.-C. Arisi, R. Kettunen, L. Jouanin, C. Foyer, Photoinhibition of photosystem II in tobacco plants overexpressing glutathione reductase and poplars overexpressing superoxide dismutase, Physiol. Plant. 105 (1999) 409–416.

[32] Y. Yamaguchi, Y. Yamamoto, H. Ikegawa, H. Matsumoto, Protective effects of glutathione on the cytotoxicity caused by a combination of aluminium and iron in suspension-cultured tobacco cells, Physiol. Plant. 105 (1999) 417–422.

[33] S. Ishida, Y. Takahashi, T. Nagata, Isolation of cDNA of an auxin-regulated gene encoding a G protein beta subunit-like protein from tobacco BY-2 cells, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 11152–11156.

[34] Y. Takahashi, T. Sakai, S. Ishida, T. Nagata, Identification of auxin-responsive elements of parB and their expression in apices of shoot and root, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 6259–6263.

[35] C.M. Johnson, P.R. Stout, T.G. Broyer, A.B. Carlton, Comparative chlorine requirements of different plant species, Plant Soil 8 (1957) 337–353.

[36] E. Fischer-Schliebs, M. Drobny, E. Ball, R. Ratajczak, U. Lüttge, Expression of different V-ATPase proteolipid subunits in slow and fast growing tobacco (*Nicotiana tabacum* L.), Aust. J. Plant Physiol. 27 (2000) 639–648.

[37] R. Ratajczak, J. Richter, U. Lüttge, Adaptation of the tonoplast V-type H^+-ATPase of *Mesembryanthemum crystallinum* to salt stress, C_3-CAM transition and plant age, Plant Cell Environ. 17 (1994) 1101–1112.

[38] H. Schägger, G. von Jagow, Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa, Anal. Biochem. 166 (1987) 368–379.

[39] P. Edman, A method for the determination of the amino acid sequences in peptides, Arch. Biochem. 22 (1949) 475.
[40] N. Popov, M. Schmitt, S. Schulzek, H. Matthies, Eine störungsfreie Mikromethode zur Bestimmung des Proteingehaltes in Gewebehomogenaten, Acta Biol. Med. Ger. 34 (1975) 1441–1446.
[41] W. Orr, T.C. White, B. Iu, L. Robert, J. Singh, Characterization of a low-temperature-induced cDNA from winter *Brassica napus* encoding the 70 kDa subunit of tonoplast ATPase, Plant Mol. Biol. 28 (1995) 943–948.
[42] L. Zimniak, P. Dittrich, J.P. Gogarten, H. Kibak, L. Taiz, The cDNA sequence of the 69-kDa subunit of the carrot vacuolar H^+-ATPase, J. Biol. Chem. 263 (1988) 9102–9112.
[43] C. Matsuura-Endo, M. Maeshima, S. Yoshida, Subunit composition of vacuolar membrane H^+-ATPase from mung bean, Eur. J. Biochem. 187 (1990) 745–751.
[44] T.A. Wilkins, Vacuolar H^+-ATPase 69-kilodalton catalytic subunit cDNA from developing cotton (*Gossypium hirsutum*) ovules, Plant Physiol. 102 (1993) 679–680.
[45] M.N. Ho, R. Hirata, N. Umemoto, Y. Ohya, A. Takatsuki, T.H. Stevens, Y. Anraku, VMA13 encodes a 54-kDa vacuolar H^+-ATPase subunit required for activity but not assembly of the enzyme complex in *Saccharomyces cerevisiae*, J. Biol. Chem. 268 (1993) 18286–18292.
[46] Z. Zhou, S.B. Peng, B.P. Drider, C. Slaughter, X.S. Xie, D.K. Stone, Molecular characterisation of the 50-and 57-kDa subunits of the bovine vacuolar proton pump, J. Biol. Chem. 273 (1998) 5878–5884.
[47] N. Tavakoli, C. Eckerskorn, D. Golldack, K.-J. Dietz, Subunit C of the vacuolar H^+-ATPase of *Hordeum vulgare*, FEBS Lett. 456 (1999) 68–72.
[48] K.S. Schumacher, D. Vafeados, M. McCarthy, H. Sze, T.A. Wilkins, J. Chory, The Arabidopsis det3 mutant role for the vacuolar H^+-ATPase in plant growth and development, Genes Dev. 13 (1999) 3257–3270.
[49] C. Bauerle, M.N. Ho, M.A. Lindorfer, T.H. Stevens, The *Saccharomyces cerevisiae* VMA6 gene encodes the 36-kDa subunit of the vacuolar H^+-ATPase membrane sector, J. Biol. Chem. 268 (1993) 12749–12757.
[50] L. Temesvari, J. Rodriguez-Paris, J. Bush, T.L. Steck, J. Cardelli, Characterization of lysosomal membrane proteins of *Dictyostelium discoideum*. A complex population of acidic integral membrane glycoproteins, Rab GTP-binding proteins and vacuolar ATPase subunits, J. Biol. Chem. 269 (1994) 25719–25727.
[51] S.Y. Wang, Y. Moriyama, M. Mandel, J.D. Hulmes, Y.C. Pan, W. Danho, H. Nelson, N. Nelson, Cloning of cDNA encoding a 32-kDa protein. An accessory polypeptide of the H^+-ATPase from chromaffin granules, J. Biol. Chem. 263 (1988) 17638–17642.
[52] B. van Hille, M. Vanek, H. Richener, J.R. Green, G. Bilbe, Cloning and tissue distribution of subunits C, D and E of the human vacuolar H^+-ATPase, Biochem. Biophys. Res. Commun. 197 (1993) 15–21.
[53] C. Kluge, D. Golldack, K.-J. Dietz, Subunit D of the vacuolar H^+-ATPase of *Arabidopsis thaliana*, Biochim. Biophys. Acta 1419 (1999) 105–110.
[54] K.-J. Dietz, B. Arbinger, cDNA sequence and expression of subunit E of the vacuolar H^+-ATPase in the inducible crassulacean acid metabolism plant *Mesembryanthemum crystallinum*, Biochim. Biophys. Acta 1281 (1996) 134–138.
[55] W. Kim, T.A. Wilkins, Isolation of a vacuolar H^+-ATPase subunit E cDNA from developing cotton (*Gossypium hirsutum*) fibers (Accession No. AF009338) (PGR97-126), Plant Physiol. 115 (1997) 314.
[56] K.-J. Dietz, B. Hollenbach, J. Arnold, Nucleotide sequences of subunit E of the vacuolar proton-ATPase of *Spinacia oleracea* (accession no. X96785) and *Arabidopsis thaliana* (accession no. X92117) (PGR96-037), Plant Physiol. 111 (1996) 652.
[57] F.M. Lagerwerf, M. van de Weert, W. Heerma, J. Haverkamp, Identification of oxidized methionine in peptides, Rap. Commun. Mass Spectrom. 10 (1996) 1905–1910.
[58] M. Schnölzer, W.D. Lehmann, Identification of modified peptides by metastable fragmentation in MALDI mass spectrometry, Int. J. Mass Spectrom. Ion Process. 169/170 (1997) 263–271.
[59] T. Fujiwara, A. Kawai, F. Shimizu, H. Hirano, S. Okuno, S. Takeda, K. Ozaki, Y. Shimada, M. Nagata, T. Watanabe, A. Takaichi, Y. Nakamura, S. Shin, Cloning, sequencing and expression of a novel cDNA encoding human vacuolar ATPase (14-kDa subunit), DNA Res. 2 (1995) 107–111.
[60] S.-B. Peng, B.P. Crider, S.J. Tsai, X.S. Xie, D.K. Stone, Identification of a 14-kDa subunit associated with the catalytic sector of clathrin-coated vesicle H^+-ATPase, J. Biol. Chem. 271 (1996) 3324–3327.
[61] L.A. Graham, K.J. Hill, T.H. Stevens, VMA7 encodes a novel 14-kDa subunit of the *Saccharomyces cerevisiae* vacuolar H^+–ATPase complex, J. Biol. Chem. 269 (1994) 25974–25977.
[62] H. Rehm, Der Experimentator: Proteinbiochemie, Gustav-Fischer-Verlag, Stuttgart, 1997.
[63] C. Bremberger, H.-P. Haschke, U. Lüttge, Separation and purification of the tonoplast ATPase and pyrophosphatase from plants with constitutive and inducible Crassulacean acid metabolism, Planta 175 (1988) 465–470.
[64] Y. Kawamura, K. Arakawa, M. Maeshima, S. Yoshida, Tissue specificity of E subunit isoforms of plant vacuolar H^+-ATPase and existence of isotype enzymes, J. Biol. Chem. 275 (2000) 6515–6522.
[65] H. Nelson, S. Mandiyan, N. Nelson, A bovine cDNA and a yeast gene (VMA8) encoding the subunit D of the vacuolar H^+-ATPase, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 497–501.
[66] D. Sakai, H.-S. Tong, C. Minkin, Osteoclast molecular phenotyping by random cDNA sequencing, Bone 17 (1995) 111–119.
[67] R. Wilson, R. Ainscough, K. Anderson, C. Baynes, M. Berks, J. Bonfield, J. Burton, M. Connell, T. Copsey, J. Cooper, A. Coulson, M. Craxton, S. Dear, Z. Du, R. Durbin, A. Favello, A. Fraser, L. Fulton, A. Gardner, P. Green, T. Hawkins, L. Hillier, M. Jier, L. Johnston, M. Jones, J. Kershaw, J. Kirsten, N. Laisster, P. Latreille, J. Lightning, C. Lloyd, B. Mortimore, M. O'Callaghan, J. Parsons, C. Percy, L. Rifken, A. Roopra, D. Saunders, R. Shownkeen, M. Sims, N. Smaldon, A. Smith, M. Smith, E. Sonnhammer, R. Staden, J. Sulston, J. Thierry-Mieg, K. Thomas, M. Vaudin, K. Vaughan, R. Waterson, A. Watson, L. Weinstock, J. Wilkinson-Sproat, P. Wohldman, 2.2 Mb of continuous nucleotide sequence from chromosome III of *C. elegans*, Nature 368 (1994) 32–38.
[68] C.J. Bult, O. White, G.J. Olsen, L. Zhou, R.D. Fleischmann, G.G. Sutton, J.A. Blake, L.M. Fitzgerald, R.A. Clayton, J.D. Gocayne, A.R. Kerlavage, B.A. Dougherty, J.-F. Tomb, M.D. Adams, C.I. Reich, R. Overbeek, E.F. Kirkness, K.G. Weinstock, J.M. Merrick, A. Glodek, J.L. Scott, N.S.M. Geoghagen, J.F. Weidman, J.L. Fuhrmann, D. Nguyen, T.R. Utterback, J.M. Kelley, J.D. Peterson, P.W. Sadow, M.C. Hanna, M.D. Cotton, K.M. Roberts, M.A. Hurst, B.P. Kaine, M. Borodovsky, H.-P. Klenk, C.M. Fraser, H.O. Smith, C.R. Woese, J.C. Venter, Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*, Science 273 (1996) 1058–1073.
[69] D.R. Smith, L.A. Doucette-Stamm, C. Deloughery, M.-H. Lee, J. Dubois, T. Aldredge, R. Bashirzadeh, D. Blakely, R. Cook, K. Gilbert, D. Harrison, L. Hoang, P. Keagle, W. Lumm, B. Pothier, D. Qiu, R. Spadafora, R. Vicare, Y. Wang, J. Wierzbowski, R. Gibson, N. Jiwani, A. Caruso, D. Bush, H. Safer, D. Patwell, S. Prabhakar, S. McDougall, G. Shimer, A. Goyal, S. Pietrovski, G.M. Church, C.J. Daniels, J.-I. Mao, P. Rice, J. Nolling, J.N. Reeve, Complete genome sequence of *Methanobacterium thermoautotrophicum* delta H: functional analysis and comparative genomics, J. Bacteriol. 179 (1997) 7135–7155.