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Short sequence-paper

Subunit D of the vacuolar H^+-ATPase of *Arabidopsis thaliana*[1]

Christoph Kluge, Dortje Golldack, Karl-Josef Dietz *

Department of Physiology and Biochemistry of Plants, Faculty of Biology – W5, University of Bielefeld, 33501 Bielefeld, Germany

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Abstract

A 1034 bp cDNA encoding the full length sequence of subunit D of the vacuolar H^+-ATPase was cloned from *Arabidopsis thaliana*. The open reading frame of the cDNA clone *vatpD* contains 780 bp and codes for a protein of 29.1 kDa with a p*I* of 9.52. Structural predictions show similarities to subunit γ of the F-ATP synthases. Identity between subunit D of the vacuolar H^+-ATPase of *A. thaliana* and subunits D from other eukaryotic organisms is in the range of 57% (*Bos taurus*) to 48% (*Candida albicans*). Hybridization of genomic DNA with *vatpD* indicates the existence of one gene copy of subunit D in *A. thaliana*. Northern blot hybridization and in situ hybridization showed expression of *vatpD* in all cell types. The expression of subunit D was not modified by salt stress or abscisic acid treatment in *A. thaliana*.

Keywords: *Arabidopsis thaliana*; ATPase; cDNA; In situ hybridization; Leaf; Subunit D; Root; Vacuole

V-Type H^+-ATPases of higher plants pump protons from the cytoplasm into the vacuole, the endoplasmic reticulum, Golgi and lysosomal vesicles at the expense of ATP and are, among other functions, involved in driving the secondary active transport of ions and metabolites across the tonoplast [1]. Energization of the tonoplast is of central importance for metabolic processes such as cell expansion, cytoplasmic ion and pH homeostasis and detoxification. The quaternary structure of the V-type H^+-ATPase enzyme shows similarities to that of the ATP synthesizing F-ATPase of mitochondria and chloroplasts [2]. The V-ATPase is composed of a V_1 domain peripherically located at the cytoplasmic side of the tonoplast and a membrane spanning V_0 domain. The V_1 domain consists of a catalytically and regulatory active 'head' assembled from subunits A and B in a threefold symmetry. The 'head' is connected to the V_0 domain by a stalk composed of at least five different subunits. Apart from the fact that the subunit composition of the stalk structure may vary in dependence of the plant species and its physiological state [3], there is convincing evidence that at least five subunits referred to as C, D, E, F, and G [2,3] are essential elements of the stalk structure. In order to understand the function of the stalk, a characterization of the subunits at the molecular level is required. From higher plants, DNA and protein sequence information of stalk subunits is available only for subunit E for instance of *Arabidopsis thaliana*, *Hordeum vulgare* and *Mesembryanthemum crystallinum* [4,5] and subunit G of tobacco [6]. Therefore, this work was initiated to obtain additional sequence information on stalk subunits of higher plants.

* Corresponding author. Fax: +49 (521) 1066039; E-mail: karl-josef.dietz@biologie.uni-bielefeld.de

[1] The cDNA of subunit D was registered at the EMBL (Hinxton, UK) under accession No. AJ225059.

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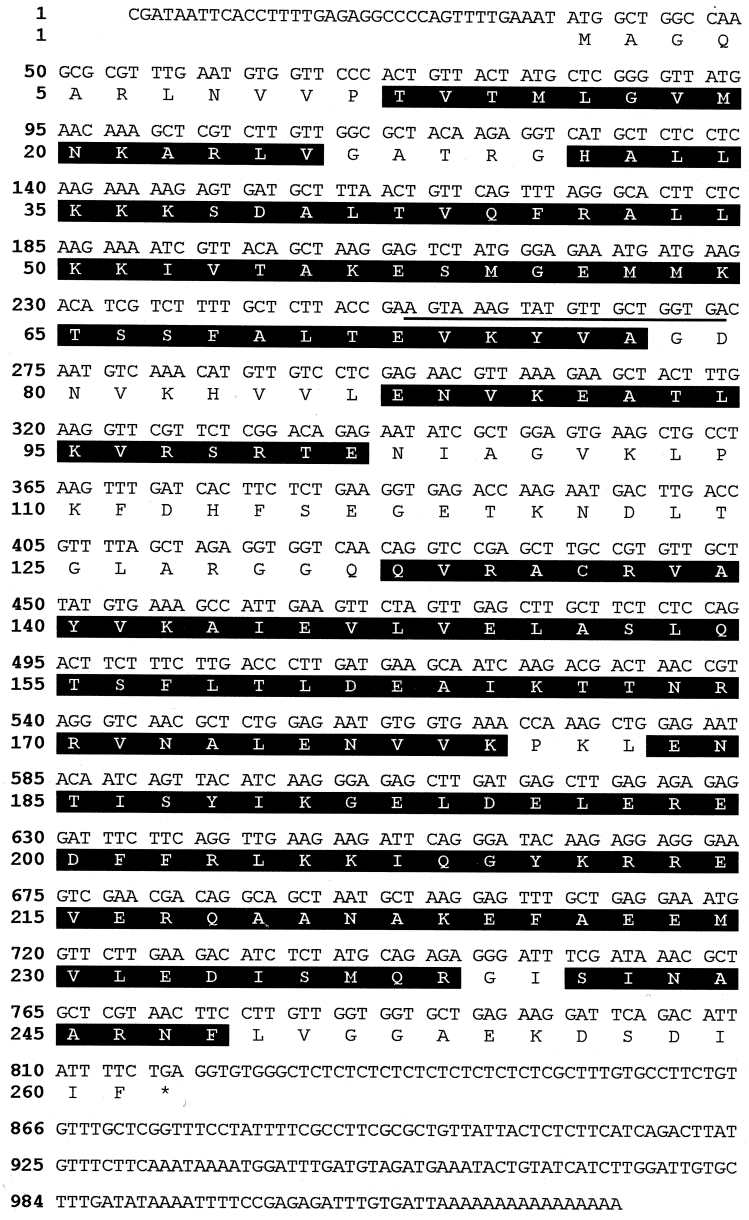

Fig. 1. Nucleotide sequence and deduced amino acid sequence of vatpD. The boxes indicate the predicted α-helices. See text for details. The nucleotide sequence of the reverse primer ATPD2 derived from the EST VBVQH07 is underlined. The forward primer ATPD1 is located upstream from the cloned sequence of vatpD.

The following approach was used to isolate the first full length cDNA encoding subunit D of the vacuolar H^+-ATPase from a plant. A cDNA sequence of 339 bp (accession No. VBVQH07) with homology to subunit D of the V-ATPase from *Bos taurus* (accession No. U11927) was identified from the EST database. By PCR with the derived oligonucleotide primers ATPD1 and ATPD2 (see legend of Fig. 1) a 330 bp fragment was obtained from *A. thaliana* cDNA. A λgt10 library of *A. thaliana* was screened with a probe of the 330 bp sequence. From 23 positive phage plaques, ten were selected for insert analysis. Eight of them hybridized at high stringency conditions to the 330 bp probe. *vatpD* contained the longest insert of 1034 bp. The open reading frame of 780 bp codes for a protein of 29.1 kDa (Fig. 1). The identity of the deduced amino acid sequence with that of subunits D of vacuolar

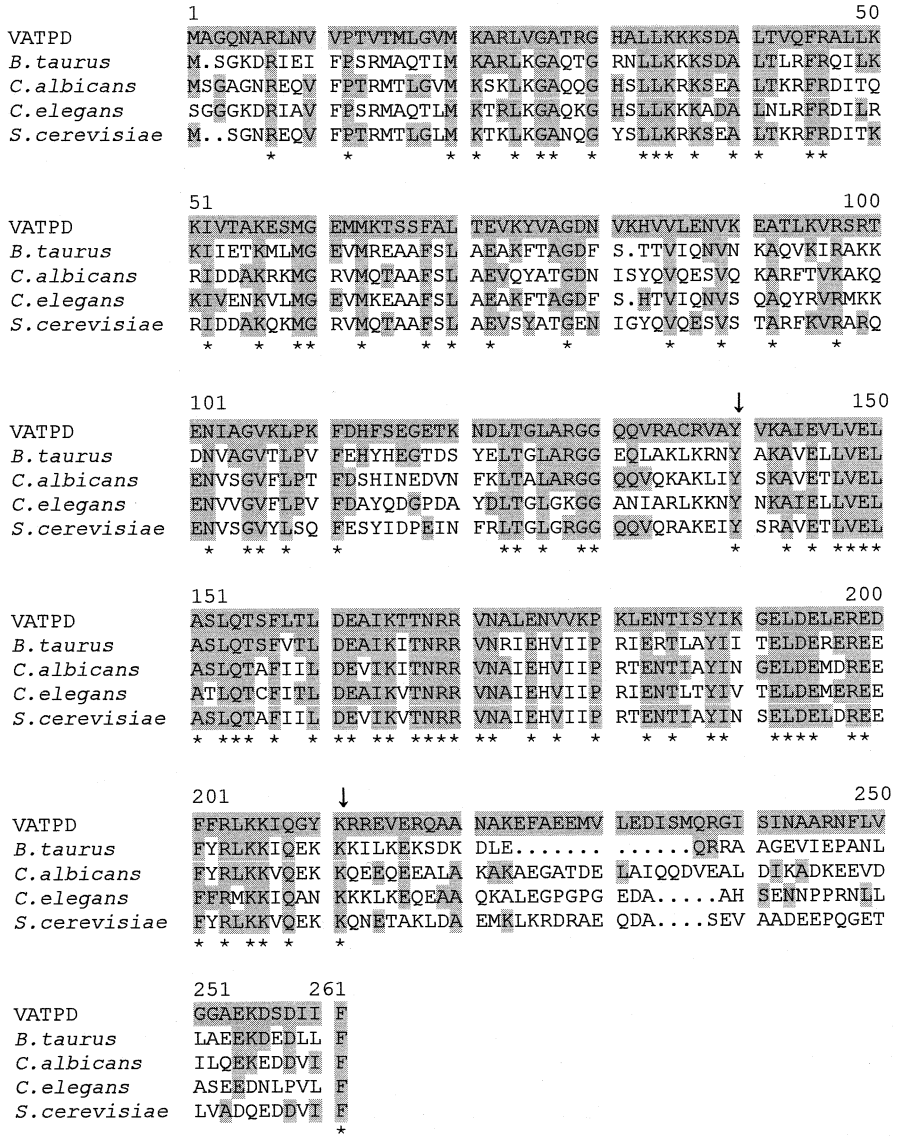

Fig. 2. Sequence alignment of subunit D of the vacuolar H^+-ATPases from various species. Amino acid sequences of subunit D from *B. taurus* (P39942), *C. albicans* (P87220), *Caenorhabditis elegans* (P34462) and *S. cerevisiae* (P32610) were aligned with the deduced amino acid sequence of VATPD. Amino acids identical with VATPD are shown in grey boxes. Stars mark the amino acids conserved throughout all species. The two arrows indicate the highly conserved region of subunit D between amino acids 139 and 211.

H^+-ATPases from other organisms ranges between 35% (*Methanococcus jannaschii*), 48% (*Candida albicans*), 53% (*Saccharomyces cerevisiae*) and 57% (*B. taurus*). Sequence similarities between the five subunit D homologues were 56% (*M. jannaschii*), 71% (*C. albicans*) 74% (*S. cerevisiae*), and 77 % (*B. taurus*) (Fig. 2).

Hydrophobicity and secondary structure of VATPD were analyzed. The hydropathy plot according to Kyte and Doolittle [7] showed no hydrophobic domains of sufficient length for a membrane spanning domain. Secondary structure was predicted for VATPD using the programme PREDATOR (EMBL). Fig. 1 indicates the sequence stretches with predicted α-helical conformation. The model suggests two helical domains with short non-helical stretches between. Similar α-helical domains are known from the γ-subunit of F-ATPases which connect the head structure with the membrane domain F_0. Such a structural similarity between subunits D

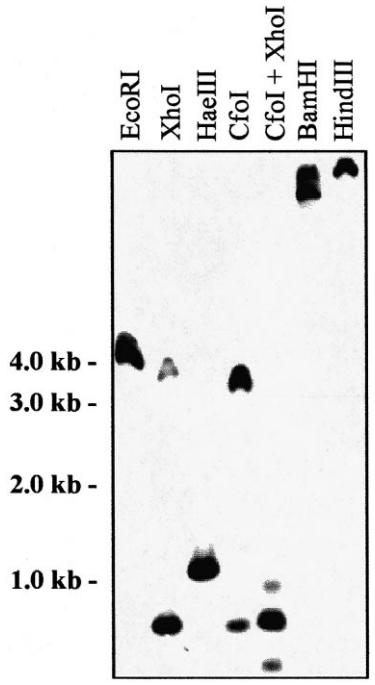

Fig. 3. Genomic Southern blot hybridized with *vatpD*. Genomic DNA from *A. thaliana* was isolated according to Gustinich et al. [14]. 10 μg of DNA were digested with restriction enzymes as indicated. The blot was hybridized with a DIG-labeled full length probe of vatpD.

and γ has been suggested before by Nelson et al. in 1995 [8]. In VATPD, there is an α-helical stretch from Thr(12) to Glu(101) with two small non-helical interruptions. A calculation based on the axial rise per amino acid residue in an α-helix of 0.15 nm suggests that this α-helix region spans at least 11.4 nm. In the γ-subunits, both the C- and N-termini are located in the head structure of the F-ATPase subunit. The length of 11.4 nm would be sufficient to extend from the top of the head to close to the membrane spanning domain V_0. Assuming a similar structure of *A. thaliana* subunit D as demonstrated for subunit γ, another α-helix would have to turn from the membrane section to the top of the head structure. VATPD has another α-helical section between Gly(129) and Gln(237) corresponding to about 16 nm. Thus, the secondary structure prediction seems to confirm the hypothesis that subunit D of V-ATPase might correspond structurally to subunit γ of F-ATPases.

A Southern analysis of genomic DNA of *A. thaliana* was performed to investigate the gene copy number of subunit D (Fig. 3). Genomic DNA was digested with different restriction enzymes and hybridized with a full length probe of vatpD. The hybridization of genomic DNA restricted with enzymes without recognition sites in the *vatpD* cDNA (*Eco*RI, *Bam*HI, and *Hin*dIII) hybridized to one band each. The result suggests that subunit D is encoded by a single copy gene in *A. thaliana*.

Expression of subunit D and its stress response was studied by Northern blot experiments. *A. thaliana* was grown in sterile culture as described in the legend of Fig. 4. The nutrient solution of one set of plants was supplemented with 100 mmol l^{-1} NaCl 1 day before harvest. The second set was grown without NaCl as control. Northern analysis was performed with a DIG-labeled probe of *vatpD* and, as a control for equal loading, with a DIG-labeled actin probe on the same blot after removal of the first probe (Fig. 4). vatpD was expressed as a 1.1 kb transcript. No effect of salt stress was seen on expression of subunit D. *A. thaliana* is salt sensitive. Salt concentrations of 100 mmol l^{-1} NaCl are highly toxic and cause necrotic lesions and finally plant death. Lower concentrations of NaCl and application of abscisic acid were also without effect on subunit D expression (not shown). The results are in contrast to salt responses of mRNAs encoding other H^+-ATP-

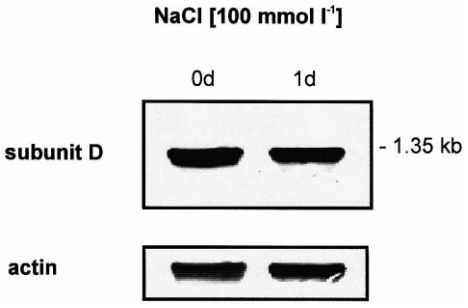

Fig. 4. Northern blot hybridization of total leaf RNA with a DIG-labeled *vatpD* probe. *A. thaliana* plants were grown on agar plates containing 1×Murashige-Skoog salts [15]. 20 days after germination, the seedlings were transferred to sterile Erlenmeyer flasks containing 10 ml 0.5×MS medium. After 24 h, the media were supplemented with 100 mmol l^{-1} NaCl or the plants were grown as a control without NaCl for 24 h. Total RNA was isolated according to Chomczynski and Sacci [16]. 10 μg of total RNA were loaded in each lane and hybridized with a DIG-labeled probe containing the complete coding sequence of *vatpD*. Hybridization and detection of the bands was performed with the DIG luminescence detection kit (Boehringer, Mannheim) according to the instructions of the manufacturer.

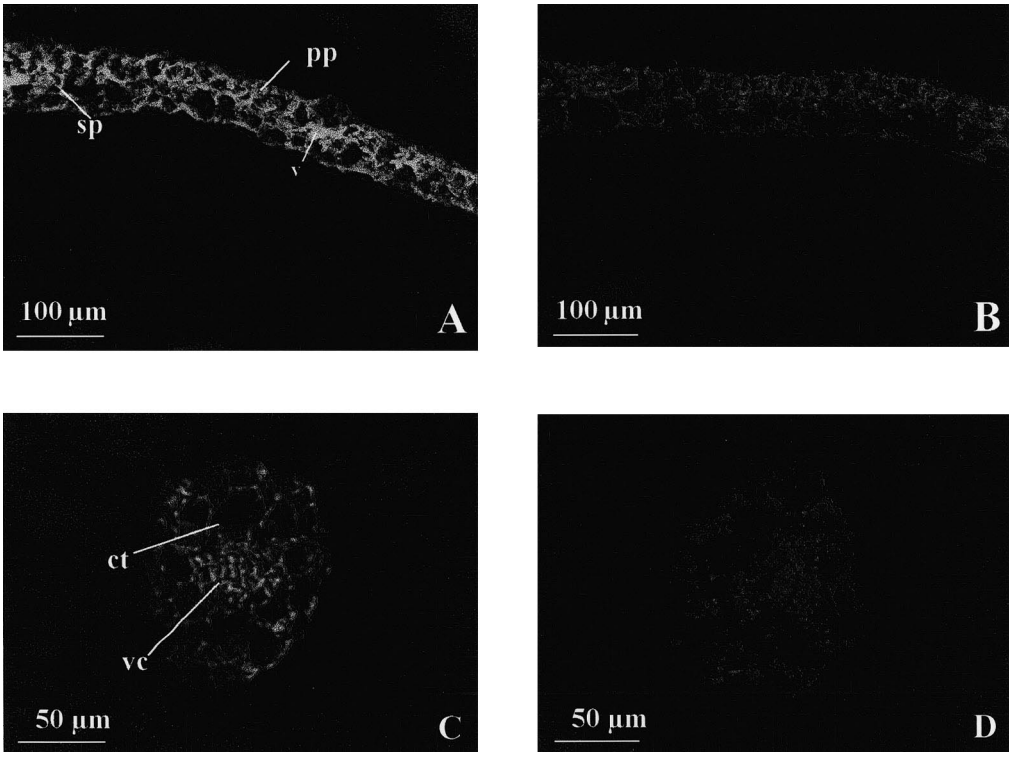

Fig. 5. In situ hybridization of *A. thaliana* leaf and root cross-sections with *vatpD*. (A) Leaf, antisense. (B) Leaf, sense. (C) Root, antisense. (D) Root, sense. v, vascular bundle; pp, palisade parenchyma; sp, spongy parenchyma; ct, cortex; vc, vascular cylinder. *A. thaliana* plants were grown for 3 weeks in sterile culture on medium containing Murashige and Skoog salts [15] with 3% sucrose and 1.2% agar, pH 5.7. Rosette leaves and roots were fixed, dehydrated, and embedded according to McKhann and Hirsch [17]. Tissue sections were hybridized with DIG-labeled (Boehringer Mannheim) antisense and sense RNA of *vatpD*. Microscopic images were obtained with an Axioskop fluorescence microscope (Zeiss, Germany) and processed by Axiovision (Zeiss, Germany) and Adobe Photoshop (Adobe Systems, USA).

ase subunits in more salt tolerant plant species such as red beet, barley and *M. crystallinum*, but also in tobacco cell culture. In these studies, application of NaCl resulted in increased activity of the vacuolar ATPase and stimulated gene expression of subunits A, B, E, and C [4,5,9–12]. The kinetics and degree of the response of expression differed, however, between the various plant systems and subunits and depended on the stress regime. Long term salt treatments of up to 3 days of *A. thaliana* with the lower concentration of 50 mmol l^{-1} did also not increase the transcript level of subunit D and may indicate distinct subunit-specific response to salt stress also in *A. thaliana* [13].

Cell specificity of *vatpD* expression was studied by in situ hybridization in rosette leaves and roots of 3 week old *A. thaliana* (Fig. 5). In leaf cross-sections, antisense *vatpD* cRNA hybridized with all cell types. Strongest signals were observed in the cells of the palisade and spongy parenchyma and in the vascular bundles. In roots, *A. thaliana* showed strongest expression of subunit D in the vascular cylinder and weaker signals in cortex and exodermis. The hybridization in all cell types observed confirms the housekeeping function of the V-H^+-ATPase.

Summarizing, this report provides the first information of subunit D structure and expression in plants. The investigation confirms findings previously reported for other subunits of the V-type H^+-ATPase, such as subunit E, that there is a significant and large degree of sequence similarity and structural conservation of subunits of the vacuolar proton pump between animals, plants, and fungi. With the knowledge of the first full length sequence of a plant subunit D, a structural analysis of the plant V-H^+-ATPase head structure will be possible, thus allowing for example to test the hypothesis of homology of subunit γ of F-ATP synthases and subunit D in V-type H^+-ATPase.

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