

Cloning of the V-ATPase subunit G in plant: functional expression and sub-cellular localization

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Abstract A 13-kDa tobacco plasma membrane protein was isolated from two-dimensional electrophoresis gels. After micro-sequencing, RT-PCR techniques and cDNA library screening allowed for the cloning of two cDNAs. These cDNAs encoded for the subunit G of the vacuolar H⁺-ATPase, the first one identified in plants. Analysis of mRNA distribution showed a maximum level in the leaves and in the stem of the apical part of the tobacco plant. Heterologous functional complementation of the yeast mutant ($\Delta vma10::URA3$) was achieved with the two cDNAs. After fractionation of microsomal membranes on linear sucrose gradient, Western blots were performed using antibodies against recombinant protein and three peaks were identified: one which comigrated with the tonoplast marker and the others at slightly higher density corresponding to endoplasmic reticulum and to plasma membrane fractions.

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Key words: H⁺-Translocating vacuolar-type ATPase; Subcellular localization; cDNA cloning; Yeast complementation; *Nicotiana tabacum*

1. Introduction

Vacuolar ATPases (V-ATPases) belong to a highly conserved family of proton-translocating ATPases present in all eucaryotic cells. They were found on a variety of cellular organelles including lysosomes, endosomes, secretory and storage vesicles, protein sorting organelles and plasma membrane, as well as on the vacuolar membrane. These enzymes are responsible for creating proton electrochemical gradients across the membrane organelles and for the maintenance of the pH homeostasis in the intracellular membrane compartment.

V-ATPases are heteromultimeric enzymes composed of a hydrophilic, catalytic V₁ complex and a hydrophobic, membrane integral V₀ complex, which together form ball and stalk structures in analogy to the ATP synthase of mitochondria, chloroplasts and bacterial membranes [1,2]. In plants, the holoenzyme has an apparent functional mass of 400–650 kDa and so far comprises 9–13 subunits [3,4]. In plant tissues, only the major subunits have already been identified at the molecular level. The gene encoding the 69-kDa subunit A was

cloned and sequenced from carrot [5] and cotton [6]. The gene encoding the 57-kDa subunit B was cloned from *Arabidopsis* [7], cotton [8] and barley [9]. For both subunits, no apparent transmembrane segment was revealed suggesting their involvement in the peripheral membrane complex. A subunit E of 31 kDa was recently cloned from barley [10]. The 16–17-kDa proteolipid, a major subunit of the membrane sector, was cloned from oat [11], cotton [12] and *Arabidopsis* [13], and the sequence analysis showed four membrane-spanning domains. Significant advances have already been done in animal and yeast V-ATPases and some other subunits were cloned in addition to subunits A, B, E and c. Subunit H, a 50–57-kDa protein, was initially cloned from yeast, where it is encoded by the VMA13 gene [14]. More recently a 14-kDa protein, subunit F, was originally cloned from insect [15] and was subsequently found in yeast [16,17]. A 13-kDa protein, subunit G, was initially cloned from yeast, where it is encoded by the VMA10 gene [18], and more recently from insect [19], bovine [20], chicken and rat [21].

Together with the plasma membrane type ATPase (P-ATPase) and the H⁺-pyrophosphatase (H⁺-PPase), the V-ATPase constitutes one of the 3 major proton pumps from plant cells. One of them, the P-ATPase, seems to have a restricted subcellular location at the plasma membrane [22]. Likewise the tonoplast is the main location for the H⁺-PPase [23] although some works point out the presence of this protein at the plasma membrane [24]. But, in contrast to these two proton pumps, it is admitted now that V-ATPases in plant, as well as in mammalian and yeast cells, can be found in a wide variety of endomembranes, such as the tonoplast [3], the Golgi apparatus [25] and clathrin-coated vesicles [26] as well as possibly in RE [25] and the plasma membrane [24]. However, except for the tonoplast where V-ATPases are involved in the regulation of vacuolar pH and play a crucial role in the control of cell expansion [12], very little is known concerning their physiological role in other membrane systems.

In this work we report the molecular cloning and the characterization of a new V-ATPase subunit in plants. A functional complementation in yeast mutant was achieved and, in addition, membrane protein localization was investigated.

2. Materials and methods

2.1. Protein purification and sequencing

Plasma membrane vesicles were purified from microsomal fractions of tobacco cells by two-phase partitioning according to [27]. Membrane proteins were separated by two-dimensional electrophoresis [28]. Gel areas corresponding to the protein of interest were digested in the gel matrix with endolysin C [29]. Peptides were separated by HPLC on a C-8 reverse-phase column (RP 300 Aquapore, 2.1 mm I.D., 100 mm length, Applied Biosystems). They were collected, con-

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centrated in vacuum and sequenced by automated Edman degradation using a gas-phase sequencer (Porton LF 3000, Beckman).

2.2. Isolation of subunit G cDNAs and sequence analysis

First-strand cDNA was synthesized from 3 µg of poly(A)⁺ RNA, extracted from tobacco cells with Superscript II reverse transcriptase (Gibco-BRL). RT-PCR were performed with degenerated oligonucleotides in sense and antisense orientations synthesized from two microsequences EIAEFRAIMEAEFQR and SPDVVQML. A 165-bp amplified fragment was obtained and was cloned in pTag vector using a commercial TA cloning kit (R&D Systems). The 165-bp PCR fragment was labeled with [α -³²P]dCTP by PCR and used as a probe to screen a tobacco leaf cDNA library in λ ZAPII vector (Stratagene). Plates were transferred to nylon membrane (Hybond N, Amersham) by double-lift procedure [30]. Hybridization was performed for 15 h at 60°C according to [31]. DNA of isolated positive clones was rescued in pBluescript phagemids after *in vivo* excision according to the supplier's instructions (Stratagene).

2.3. RNA extraction and hybridization

Total RNA was extracted by acid guanidinium thiocyanate/phenol/chloroform single step method [32]. Total RNA was denatured in 50% formamide and 2.2 M formaldehyde for 5 min at 60°C and separated in 1.25% agarose containing formaldehyde [30]. The detection of ribosomal RNA for checking loading homogeneity was performed by staining with methylene blue [33]. RNAs were hybridized with PCR amplified DIG-dUTP labeled probes, using the DIG labeling. The detection was achieved with a chemiluminescent detection system kit (Boehringer Mannheim).

2.4. Yeast mutant complementation

The yeast strain W303 1-B (Δ mal10::URA3) was kindly provided by Dr. Nathan Nelson (University of Tel Aviv, Israel). The coding regions flanked by *Bsa*I and *Bam*HI sites of the two cDNA *Nt-vag1* and *Nt-vag2* were produced by PCR as described above and cloned into the filled *Hind*III site and the *Bam*HI site of the yeast expression vector pVT-Leu (gift of D. Tessier, CRNC and S. Dequin, INRA, Montpellier). Yeast cells were transformed according to [34] and spread onto selective medium (YNB, 50 mM MES, 50 mM MOPS buffered at pH 5.5, 2% agar) with the appropriate markers (Ade, His and Trp). Selected clones were spread on a solid rich medium (YPD) buffered at pH 7.5 to achieve the functional complementation. Measurements of quinacrine uptake were achieved according to [18] using a fluorescent microscope (Nikon).

2.5. Sub-cloning and production of polyclonal antibodies

Polyclonal antibodies were raised against the recombinant protein Nt-vag1 produced in *E. coli*. The coding region of the cDNA Nt-vag1 was amplified by PCR using a 5'-primer with *Bam*HI and *Bsa*I sites and a 3'-primer with a *Bam*HI site. The amplified fragment was cloned into the *Bam*HI site of the pBluescript vector and sequenced on both strands. Then *Bsa*I-*Bam*HI fragment was blunted with Klenow at *Bsa*I extremity and introduced between the *Bam*HI and the filled *Nde*I sites of the expression vector pET-3c (Novagen). This construction was introduced in *E. coli* BL21(DE3) strain. Protein expression was induced by adding 0.4 mM isopropyl β -D-thiogalactopyranoside (IPTG) to a mid-log culture. After preparation of insoluble matter, the recombinant polypeptides were solubilized and purified on two-dimensional gel as described by [28]. After CuCl₂ staining [35], acrylamide spots containing the 13-kDa overexpressed polypeptide were crushed in 1 ml of PBS. The resulting mixture was mixed with Freund's adjuvant (v/v) and used to immunize rabbits intradermally. Booster injections were given 1 and 2 months later.

2.6. Isopycnic gradient and immunodetection

Microsomal membranes were prepared from 40-day-old tobacco leaves essentially as described by [36]. Microsomes were layered on the top of a 36-ml linear 15–45% (w/v) sucrose gradient. Sucrose solutions were buffered to pH 7.2 with 1 mM Tris-MES containing 1 mM DTT. Gradients were centrifuged at 100 000 $\times g$ for 16 h and collected into 1.5-ml fractions. Protein samples were separated on denaturing 14% acrylamide gels followed by an electrotransfer onto PVDF membranes (Millipore). Membranes were immunostained with various antibodies (anti-H⁺-ATPase, 1:10 000 [37]; anti-60-kDa-V-ATPase, 1:300 [38]; anti-calreticulin, 1:2000 [39]). The detection was

performed with a chemiluminescent detection system kit (SuperSignal, Pierce).

3. Results

3.1. Isolation of *Nt-vag* cDNAs and sequence analysis

Within the course of a systematic plasma membrane protein characterization combining 2-D gel electrophoresis and protein sequencing, 2 internal sequences were initially obtained for a 13-kDa protein. These sequences showed homology to one castor bean (*Ricinus communis*) and four rice (*Oryza sativa*) ESTs. The analysis of EST sequences suggested further homology with cDNAs encoding subunit G of vacuolar-type H⁺-ATPase from *Manduca sexta* and *Saccharomyces cerevisiae*.

Degenerated oligonucleotides were designed in order to amplify a partial cDNA corresponding to the plant protein. A cDNA of 165 bp was amplified and the deduced sequence was similar to those of subunit G of V-ATPases present in data bases (data not shown). To isolate full-length clones, the 165-bp cDNA was subsequently used as hybridization probe to screen a λ Zap cDNA library from tobacco leaves.

Ten positive clones were isolated and sequenced. The length of the different cDNAs was between 552 and 810 bp. Seven clones presented an open reading frame of 333 bp encoding an identical protein of 110 amino acids. Their sequences diverged only in the 5' and 3' untranslated regions (UTR). The longest of them (810 bp) was entirely sequenced and was called *Nt-vag1* (*Nicotiana tabacum* Vacuolar ATPase subunit G₁). The complete nucleotide sequence of this cDNA and its deduced amino acid sequence are shown in Fig. 1. The molecular weight of the protein was calculated to be 12 495 Da with a *pI* of 5.94. The three other clones had an identical open reading frame of 336 bp encoding a predicted sequence of 111 amino acids. The translated sequence included one peptide identical to one of those used to design oligonucleotides whereas the other one differed by one residue (His⁹⁶) from the initial protein microsequence. The longest of them (666 bp) was entirely sequenced and called *Nt-vag2*. The complete nucleotide sequence of this cDNA and its deduced amino acid sequence are shown in Fig. 1. The molecular weight of the protein was calculated to be 12 520 Da with a *pI* of 5.94.

Nt-vag1 and *Nt-vag2* were 86% identical in their coding regions and the predicted proteins were 85% identical and 94% similar. In contrast, their 3'-UTR regions were strongly divergent with 28% identity in the overlapping regions. Most of the sequence differences in the coding region were attributed to third base degeneracy. The presence of an additional amino acid (Ser⁷) in *Nt-vag2* was correlated with the existence of a potential consensus sequence for myristoylation, which was, absent in *Nt-vag1*. Both open reading frames (ORFs) were likely to be complete since the sequence around the initiation codon is similar to ATG initiation consensus in plants [40].

The alignment of *Nt-vag1* and *Nt-vag2* with the V-ATPases characterized in different organisms (yeast, insect and mammals) showed a high degree of conservation between all of them (Fig. 2). In general, residues of the N-terminal half of the proteins were more conserved than those of the C-terminal region. Each of the sequence *Nt-vag1* and *Nt-vag2* showed rather the same degree of similarity with other V-ATPases. Whatever the organism considered, *Nt-vag1* is more homolo-

Fig. 1. Nucleotide and predicted protein sequences of tobacco cDNA *Nt-vag1* and *Nt-vag2*. Nucleotide sequences are indicated in small letters and protein sequences in capital letters. For *Nt-vag2*, dashes indicate identity with *Nt-vag1*. Translation start and stop sites are indicated in bold. Peptide sequences obtained by microsequencing are underlined.

Fig. 2. Comparison of deduced amino acid sequences of *Nt-vag1* and *Nt-vag2* genes with five full-length sequences of representative V-ATPase subunits G. Subunit G protein sequences are from: tobacco (Nt-vag1 and Nt-vag2) (this paper); rat brain (VCG1_RAT); chicken osteoclast (VCG1_CHICK); bovine brain (VCG2_BOVIN [21]); *Manduca sexta* (VCG1_MANSE [19]); *Saccharomyces cerevisiae* (VMA10 [18]). The seven proteins were aligned using the PILEUP program from UWGCG Wisconsin package. Dark and grey areas show, respectively, identical and conserved amino acids.

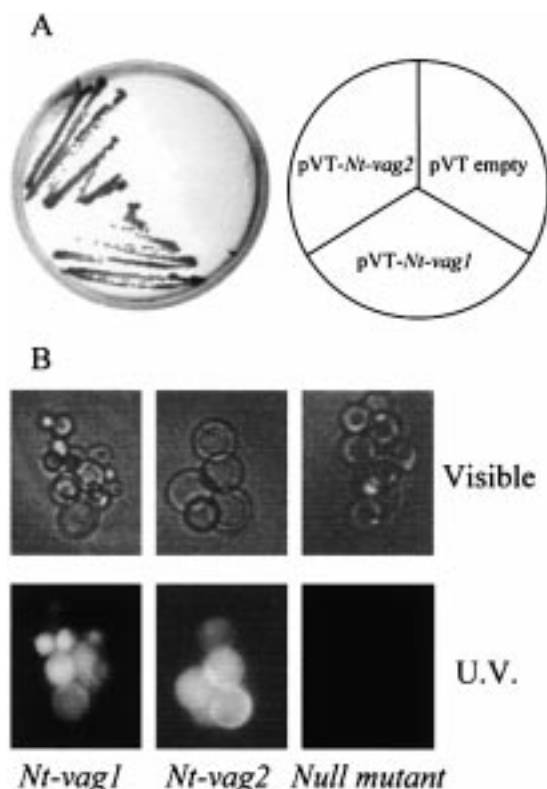


Fig. 3. Complementation of the yeast null mutant *vma10::URA3* with *Nt-vag1* and *Nt-vag2* cDNAs. A: Transformed yeast cells on YPD medium pH 7.5. B: Accumulation of quinacrine in transformed and null mutant yeast vacuoles.

gous (35% identity and 60% similarity) to the other sequences than *Nt-vag2* (32% identity and 56% similarity). This difference could be in part attributed to their divergence in the N-terminal region.

3.2. Yeast mutant complementation

In order to confirm the function of the proteins encoded by the *Nt-vag1* and *Nt-vag2* cDNAs, a yeast mutant complementation approach was engaged. The used yeast null mutant *vma10::URA3* [18] failed to grow at pH 7.5 like any other mutant in which a gene encoding V-ATPase subunit has been disrupted [41]. *Nt-vag1* and *Nt-vag2* cDNAs were cloned in the yeast expression vector pVT-Leu. After transformation of the yeast null mutant *vma10::URA3* with the two constructions, colonies able to grow on minimal medium without leu-

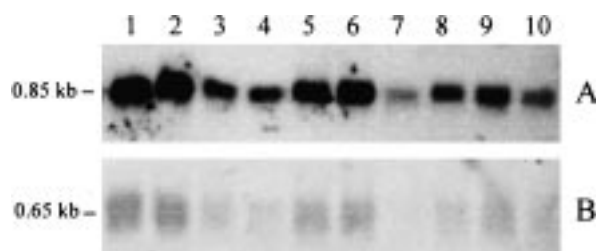


Fig. 4. Northern blot analysis of *Nt-vag1* (A) and *Nt-vag2* (B) transcripts in tobacco plants. Total RNA (20 µg/lane) was extracted from leaves of vegetative plants (1) and from different tissues of plants at floral stage: leaves from apical (2), median (3), basal zone (4); stems from apical (5), median (6), inner part of basal zone (7) and outer part of basal zone (8); floral buds (9) and roots (10).

cine were spread on YPD medium buffered at pH 7.5. In these conditions, as shown in Fig. 3A, only yeast carrying the *Nt-vag1* or *Nt-vag2* cDNAs were able to grow. To further analyze the complemented yeast mutant, the accumulation of the pH probe quinacrine was monitored in null mutant and complemented yeast vacuoles. Vacuoles of the yeast null mutant were not labeled by quinacrine while for complemented yeast an accumulation of the fluorescent probe was observed indicating a proper acidification (Fig. 3B).

3.3. Analysis of *Nt-vag1* and *Nt-vag2* mRNA expression in plants

The accumulation of *Nt-vag1* and *Nt-vag2* transcripts was analyzed by Northern blotting (Fig. 4). Total RNA was prepared from leaves of vegetative plants and from leaves, stems, floral buds and roots at floral stage. A 0.85-kb mRNA was detected with a probe corresponding to the full-length cDNA *Nt-vag1*, whatever the tissue analyzed. The abundance of the mRNA was higher in the youngest tissues of mature plants, like leaves and stems of the apical meristematic zone, than in the older part of plants. On the other hand, a band at 0.65 kb was detected with the probe specific to the full-length cDNA *Nt-vag2*. The pattern of *Nt-vag2* expression in the plant was similar to that of *Nt-vag1*, but revealed a lower accumulation level.

3.4. Subcellular location

To determine the subcellular localization of the 13-kDa protein, tobacco microsomal membranes were fractionated on an isopycnic sucrose gradient (15–45%, w/v) and the fractions were analyzed by Western blotting (Fig. 5). The tonoplast V-ATPase, as identified by the use of monoclonal antibodies specific to the 60-kDa subunit [38], peaked at a density equivalent to 29% sucrose. Antibodies raised against an H⁺-ATPase [37] were used to probe the plasma membrane and an immunoreactive peak was revealed at a density equivalent to 41% sucrose. The endoplasmic reticulum marker calreticulin [39] presented a maximum at a density equivalent to 36% sucrose. The polyclonal antibodies raised against the recombinant subunit G, revealed three peaks: one at a density equivalent to 30% sucrose, a second one at a density equivalent to

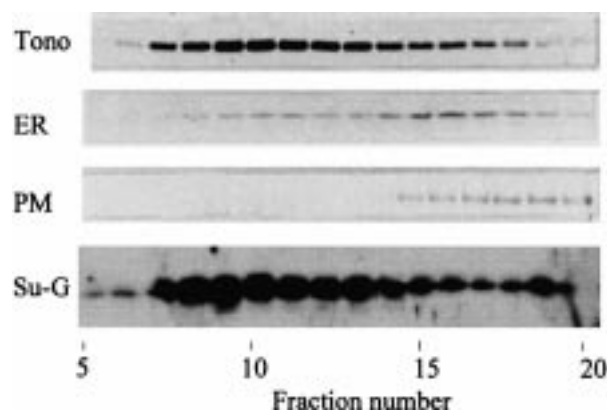


Fig. 5. Immunological distribution of proteins present in linear sucrose gradient fractions of tobacco leaf microsomes. Fractions were subjected to SDS-PAGE and Western blot analysis, and were probed with anti-subunit G (Su-G) and antibody markers for the plasma membrane (PM; anti-H⁺-ATPase), the tonoplast (tono; anti-V-ATPase subunit B) and the endoplasmic reticulum (ER; anti-calreticulin).

36% sucrose and the third one at a density equivalent to 41% sucrose. These results suggested that although the subunit G of V-ATPase of tobacco leaves was mainly located at the vacuolar membrane, it may also be associated to the endoplasmic reticulum and to the plasma membrane.

4. Discussion

Here we report the cDNA cloning of two related subunits G of V-ATPase in tobacco that constitute the first subunit G identified in plants until now.

Starting from a protein purified from plasma membrane and partially microsequenced, degenerated oligonucleotides were used to amplify a partial cDNA and screened a cDNA library from tobacco leaves. Simultaneously to a clone specific to the purified protein, *Nt-vag1*, another one, *Nt-vag2*, was isolated encoding a near identical protein (Fig. 1). *Nt-vag1* and *Nt-vag2* encode very similar hydrophilic proteins of respectively 110 and 111 amino acids, with a calculated molecular weight of about 12.5 kDa and an acidic isoelectric point of 5.94. *Nt-vag1* and *Nt-vag2* share high homology (about 60%) with the 13-kDa subunits G already described for yeast [18], tobacco hornworm [19], bovine, rat and chicken [21]. But in opposition to these proteins which are basic proteins with an isoelectric point around 9, *Nt-vag1* and *Nt-vag2* are acidic proteins. The significance of this specificity is unknown, but inasmuch as the two clones are functional in yeast, this can be considered as a plant specific feature. The functional demonstration of the two cDNAs was achieved by yeast mutant complementation. The two cDNAs, *Nt-vag1* and *Nt-vag2*, restore the capacity of the yeast mutant $\Delta\text{vma10::URA3}$ to grow on a selective medium and the activity of the V-ATPase.

One of the functions of V-ATPases in plants is to contribute, with H^+ -PPase, to create a proton electrochemical gradient across the tonoplast. This V-ATPase activity has been shown to be essential for enlargement of cells, for example, the rapid expansion of cells of cotton petals and carrot tip roots. When the expression of subunit A of V-ATPase was inhibited by antisense mRNA, altered leaf morphologies and reduced cell expansion were observed [42]. Our results indicated that the abundance of the *Nt-vag* mRNAs were higher in the youngest tissues which were considered to be in the elongating phase (Fig. 4).

If the multiple location of the H^+ -pumping ATPases of the V-type is well admitted in insects and animals, the presence of the V-ATPases on the plant plasma membrane is not as widely recognized. Our results suggest that the subunit G was localized on two different endomembranes such as tonoplast and ER, and on plasma membrane in various proportions. These results are in total accordance to those of Herman et al. [25] on the presence of the V-ATPase subunits A and B in endomembrane fractions from oat root tips. These authors suggested that the V-ATPases are associated with ER, Golgi or Golgi-derived vesicles, provacuoles, tonoplast and plasma membrane. Using a post-embedding immunogold technique and a polyclonal antiserum raised against the holoenzyme of the V-ATPase, Robinson et al. [24] detected the presence of a vacuolar-type H^+ -ATPase in the plasma membrane and trans-Golgi elements of pea cotyledons.

In conclusion, our results demonstrate the presence in plants of subunit G for the V-ATPase. This subunit differs from those previously characterized in yeast, insects and

mammals by its pI, but nevertheless remains able to complement defective yeast. In addition it exhibits a quite complex pattern of localization. This suggests that plant V-ATPases show distinctive features by comparison to animal V-ATPases, but share with them multiple roles within the cell.

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