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Isolation and Characterization of cDNAs Encoding the Vacuolar H+-Pyrophosphatase of *Beta vulgaris*[1]

Yongcheol Kim, Eugene J. Kim, and Philip A. Rea*
Plant Science Institute, Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6018

The H⁺-pyrophosphatase (V-PPase) of plant vacuolar membranes catalyzes the electrogenic translocation of H⁺ from the cytosol to vacuole lumen and, in parallel with the vacuolar H⁺-ATPase located in the same membrane, establishes the inside-acid, inside-positive H⁺-electrochemical potential difference responsible for energizing the H⁺-coupled transport of solutes into the vacuole. The results of previous investigations suggest that the gene encoding the substrate-binding subunit of the V-PPase is present in a single copy in the genome of *Arabidopsis thaliana* (V. Sarafian, Y. Kim, R.J. Poole, P.A. Rea [1992] Proc Natl Acad Sci USA 89: 1775–1779), but it is not known whether the situation in *Arabidopsis* is typical of most vascular plants. With the objective of assessing the general applicability of this finding and acquiring sequence data for structure-function analyses of the enzyme from *Beta vulgaris*, we have sought to isolate cDNAs encoding the V-PPase from this organism by screening a *Beta* cDNA library constructed in λZAP with the *Arabidopsis* cDNA insert (AVP) encoding the V-PPase. The results of these investigations demonstrate that at least two genes encode the V-PPase in *Beta*. Restriction and sequence analyses of the cDNAs from *Beta* reveal two classes, designated *BVP1* and *BVP2*. *BVP1* and *BVP2* encode closely related but distinct polypeptides with computed masses of 80,550 and 80,000 D, respectively, exhibiting 88% identity with each other and 89% identity with the corresponding polypeptide from *Arabidopsis*. The nucleotide sequences of *BVP1* and *BVP2*, on the other hand, are 70% identical within their coding regions but less than 28 and 53% identical within their respective 5′ and 3′ noncoding regions. Southern analyses of *Beta* genomic DNA confirm that two genes encode the V-PPase, and northern analyses of polyadenylated RNA isolated from a range of tissue types and probed with RNAs transcribed from the 3′ noncoding sequences of *BVP1* or *BVP2* indicate that both genes are expressed in the intact plant. On the basis of these findings and the recent demonstration of the sufficiency of the substrate-binding polypeptide, alone, for all of the known catalytic functions of the V-PPase (E.J. Kim, R.-G. Zhen, P.A. Rea [1994] Proc Natl Acad Sci USA [91: 6128–6132]), the two cDNA species isolated from *Beta* are concluded to encode variants, possibly isoforms, of the enzyme.

PPases (EC 3.6.1.1) are ubiquitous enzymes that catalyze the hydrolysis of PPi to Pi. The bulk of cellular PPase activity in most organisms is soluble and acts to mediate the dissipative hydrolysis of PPi. Soluble PPases thereby maximize the energy yield from the pyrophosphorylytic cleavage of nucleoside triphosphates and facilitate reactions that would otherwise be thermodynamically unfavorable (Voet and Voet, 1990). In some organisms, however, membrane-associated, energy-conserving PPases are also found (Baltscheffsky and Baltscheffsky, 1993). Notable examples are the reversible H⁺-translocating PPase of chromatophores from the purple, nonsulfur bacterium *Rhodospirillum rubrum* (Nyren et al., 1991), the membrane-linked PPases of animal and plant mitochondria (Lundin et al., 1991), and the V-PPase of plants (Rea et al., 1992b; Rea and Poole, 1993). The capacity of the mitochondrial PPase for ion transport has yet to be demonstrated directly, but the enzymes from *Rhodospirillum* and plants are PPi-energized pumps active in the primary translocation of H⁺ (Baltscheffsky and Baltscheffsky, 1993) and H⁺ and/or K⁺, respectively (Davies et al., 1992; Rea and Poole, 1993).

The importance of PPi as an energy source for vacuolar function, in particular, and plant cell metabolism, in general, is evident at several levels. First, the V-PPase is a universal and abundant component of plant vacuolar membranes (Rea and Poole, 1993), capable of generating a steady-state transtonoplast H⁺-electrochemical potential difference of similar or greater magnitude than the V-ATPase (EC 3.6.1.3) on the same membrane (Pope and Leigh, 1987; Johannes and Felle, 1989; Maeshima and Yoshida, 1989; Rea et al., 1992a). Second, the vacuole frequently constitutes 40 to 99% of the total intracellular volume of a mature plant cell. As such, the cytosol is often a thin interfacial zone delimited and regulated by the plasma membrane and tonoplast. Third, the vacuolar membrane is implicated in a broad spectrum of physiological processes, all of which depend on the maintenance of a transtonoplast H⁺ gradient. Examples include cytosolic pH stasis, compartmentation of regulatory Ca^{2+}, sequestration of otherwise toxic ions such as Na^+, cytosolic elimination of alkaloids, turgor regulation, and nutrient storage and retrieval. Hand in hand with elucidation of the role played by PPi as an energy source for a number of other metabolic reactions in plant cells, specifically cytosolic Suc mobilization via Suc synthase (Huber and Akazawa, 1986; Black et al., 1987) and glycolysis via PPi:Fru-6-P 1-phosphotransferase (Black et al., 1987; Dennis and Greyson, 1987), recognition of the pivotal role played by the V-PPase in the establishment

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* Corresponding author; fax 1-215-898-8780.

Abbreviations: AVP, cDNA encoding *Arabidopsis* vacuolar H⁺-pyrophosphatase; BVP, cDNA encoding *Beta* vacuolar H⁺-pyrophosphatase; PPase, pyrophosphatase; V-ATPase, vacuolar H⁺-ATPase; V-PPase, vacuolar H⁺-pyrophosphatase.

of transmembrane solute gradients has reinforced the notion that plants possess parallel metabolic pathways that use either PPi or nucleotides as alternate energy sources.

Progress toward definition of the structure and function of the V-PPase, which belongs to a new category of ion translocase (Rea et al., 1992b), has been rapid. Recent accomplishments include identification (Britten et al., 1989; Rea et al., 1992a) and purification (Britten et al., 1989; Maeshima and Yoshida, 1989; Sarafian and Poole, 1989; Rea et al., 1992a) of the major substrate (Mg_2PPi; Leigh et al., 1992; Baykov et al., 1993) binding subunit, isolation and characterization of cDNAs encoding this subunit (Sarafian et al., 1992; Tanaka et al., 1993), and reconstitution of partially purified preparations of the enzyme to yield proteoliposomes competent in PPi-dependent H^+-translocation (Britten et al., 1992).

However, although the results of these investigations and the recent success in heterologously expressing a single cDNA species encoding the substrate-binding subunit of the V-PPase from the vascular plant *Arabidopsis thaliana* in the yeast *Saccharomyces cerevisiae* to generate enzyme active in K^+-stimulated, PPi-dependent H^+-translocation (Kim et al., 1994) demonstrate that one polypeptide species is sufficient for the assembly of functional enzyme, it is not known whether isoforms of this one subunit exist. On the one hand, most primary ion translocases are known to exist as multiple isoforms. For instance, in mammals there are at least five isoforms of the Na^+/K^+-ATPase (Verrey et al., 1989), in vascular plants there are more than 10 isoforms of the plasma membrane H^+-ATPase (Harper et al., 1990; DeWitt et al., 1991), and in both animals and plants several isoforms of the V-ATPase have been identified (Sze et al., 1992). On the other hand, in the two organisms from which the V-PPase has been cloned, *Arabidopsis* and *Hordeum vulgare*, only a single cDNA species from each has been identified to date (Sarafian et al., 1992; Tanaka et al., 1993). The apparent absence of multiple cDNAs encoding the V-PPase of *Arabidopsis* and *Hordeum* does not preclude the existence of isoforms, albeit divergent ones, in these organisms, but it does leave unanswered the question of whether isoforms remain to be discovered. Thus, in an attempt to delineate the potential functional repertoire of the V-PPase and as a prelude to protein chemical topological studies of the enzyme in an inherently sided membrane preparation—isolated, intact vacuoles from *Beta vulgaris* storage root, which can be prepared mechanically in high yield by standard procedures (Leigh and Branton, 1976)—the cloning of cDNAs encoding the V-PPase from this organism is reported. Contrary to the situation in *Arabidopsis*, these cDNAs fall into two classes: *BVP1* and *BVP2*. By showing that both *BVP1* and *BVP2* encode the substrate-binding subunit, we provide the first evidence that the V-PPase may be subject to regulatory and/or functional bifurcation.

MATERIALS AND METHODS

Materials

Beta vulgaris L. cv Detroit Dark (red beet) was the plant material used in these investigations. Restriction enzymes and sequencing materials were purchased from BRL, New England Biolabs, and Promega; [α-^{35}S]dATP, for sequencing, and ^{32}P-labeled nucleotides were obtained from Amersham Corp. and ICN Radiochemicals, respectively; λZAP, pBluescript, λ packaging extracts, and *Escherichia coli* strains were bought from Stratagene; and the Sequenase sequencing kit was from United States Biochemical.

Construction and Screening of *Beta* λZAP cDNA Library

A *B. vulgaris* cDNA library constructed in λZAP was prepared from poly(A)$^+$ RNA purified from 7-d-old, dark-grown seedlings. Total RNA was extracted by the phenol/SDS method (Ausubel et al., 1987), poly(A)$^+$ RNA was purified by oligo(dT)-cellulose chromatography (Sambrook et al., 1989), and double-stranded cDNA was synthesized using a UNI-ZAP XR cDNA synthesis kit (Stratagene). The reverse transcripts were size fractionated, and the 5' and 3' termini of those cDNAs fractionating at greater than 0.5 kb were ligated to *Eco*RI and *Xho*I adapters. After directional ligation of the inserts into λZAP and packaging, the library was amplified once to yield 5×10^9 plaque-forming units/mL of which more than 98% contained inserts.

Approximately 120,000 plaques from the λZAP cDNA library were initially screened with random-primed *Arabidopsis* cDNA insert AVP-1 (Sarafian et al., 1992) at high stringency (Sambrook et al., 1989). Five independent positive clones were obtained from this screen and subsequently found to correspond to pBVP2. The cDNA insert of one of the pBVP2 clones was then used to screen an additional 100,000 plaques from which an additional three pBVP2 and two pBVP1 clones were obtained. The clones were colony purified, and their cDNA inserts were subcloned into the *Xba*I/*Xho*I sites of pBluescript. Both strands of the longest inserts from each class of cDNA, henceforth referred to as "*BVP1*" and "*BVP2*," were sequenced.

Northern Blot Hybridizations

Total RNA was extracted from various tissues of 7-d and 1- and 7-month-old plants of *B. vulgaris* (Ausubel et al., 1987), and poly(A)$^+$ RNA was extracted and purified (Sambrook et al., 1989). After size fractionation by electrophoresis on 1% agarose gels containing 0.63 M formaldehyde, the poly(A)$^+$ RNA was blotted onto nylon membrane filters (Nytran-45, Schleicher & Schuell). The filters were prehybridized at 42°C for 2 h in prehybridization solution containing 50% (v/v) formamide, 5× SSPE (1× SSPE is 0.15 M NaCl, 10 mM NaH_2PO_4, pH 7.4, 1 mM Na_2EDTA), 1× Denhardt's solution (50× Denhardt's solution is 1% PVP, 1% BSA, and 1% Ficoll-400), and 0.1 mg/mL denatured, sonicated salmon sperm DNA. The filters were hybridized in the same solution containing ^{32}P-labeled riboprobe at 65°C, washed three times with 1× SSPE/0.5% SDS at 65°C, and finally washed with 0.1× SSPE/0.5% SDS at 60°C. The filters were exposed to Kodak X-Omat x-ray film with intensifying screens for 12 to 18 h at −85°C.

[α-^{32}P]CTP-labeled RNA transcribed from the 2.1-kb *Hin*dIII fragment of *BVP1* was used as a nonspecific BVP probe, and RNAs transcribed from the 3' noncoding sequences of *BVP1* and *BVP2* were used as gene-specific probes.

The 2.1-kb *Hin*dIII fragment of *BVP1*, which corresponds to the open reading frame, and *Eco*RI/*Xho*I fragments of *BVP1* (428 bp) or *BVP2* (398 bp), which correspond to the 3′ noncoding sequences of the two cDNAs, were gel purified and ligated into pBluescript. The orientation and sequence of each construct was confirmed by sequencing both ends of the inserts, and riboprobes were transcribed from the antisense strands of the templates using T7 or T3 RNA polymerase (Sambrook et al., 1989).

Genomic Blot Hybridizations

Genomic DNA was extracted from 7- to 10-d-old seedlings of *B. vulgaris* (Ausubel et al., 1987). Samples (4 μg) of the DNA were digested with restriction enzyme, electrophoresed on 0.8% agarose gels, and transferred to nylon (Nytran-45) membrane filters. The filters were prehybridized in 6× SSPE, 10× Denhardt's solution, and 0.1 mg/mL denatured salmon sperm DNA at 55°C and hybridized at 55°C in 50% formamide, 6× SSPE, 0.5% SDS, and 0.1 mg/mL denatured, sonicated salmon sperm DNA containing ^{32}P-labeled, random-primed 2.1-kb *Hin*dIII *BVP1* fragment. The filters were successively washed in 1× SSPE/1.0% SDS and 0.1 × SSPE/1.0% SDS at 65°C before exposure to x-ray film as described above for the northern blot hybridizations.

DNA Sequencing

DNA was sequenced by the dideoxynucleotide chain termination method (Sanger et al., 1977) using a Sequenase II kit. A series of nested deletions from the cDNA inserts of pBVP1 and pBVP2 were obtained using a double-stranded nested deletion kit (Pharmacia). The remaining sequencing reactions were performed using custom-synthesized oligonucleotide primers.

RESULTS

A total of 10 positive clones were obtained from the screens of the *B. vulgaris* cDNA library using random-primed, ^{32}P-labeled AVP-1 cDNA (Sarafian et al., 1992) as the hybridization probe. Of these, two were identified as containing inserts corresponding to *BVP1* and eight to *BVP2* on the basis of their restriction patterns. The longest cDNA inserts (2.8–2.9 kb) from each class were subcloned into pBluescript and sequenced. The complete nucleotide sequences and predicted amino acid sequences of representatives from each class are shown in Figures 1 and 2.

The Met (ATG) codons at position 1 in the sequences of *BVP1* and *BVP2* were identified as the translation initiation sites for two reasons: (a) Only by proposing translation initiation at these sites can the N-terminal sequence obtained by Maeshima and Yoshida (1989) for the substrate-binding subunit of the V-PPase from *Vigna radiata* be accommodated by the deduced sequences of the polypeptides encoded by the two cDNA species from *Beta* (Fig. 2A). (b) The sequences contiguous with these, the first Met codons, match the consensus sequences for plant translation initiation sites (RA/CA/C AUG GC) with R (purine) at −3 and G at position +4 being the most critical (Lutcke et al., 1987). *BVP1* contains the sequence AAT ATG GGT, and *BVP2* contains the sequence AAA ATG ATT starting from position −3 (Fig. 1). Hence, the open reading frame of the cDNA insert of pBVP1 encompasses 2295 nucleotides followed by two tandem termination codons (TAA TGA) and 462 nucleotides of 3′ noncoding sequence, whereas pBVP2 comprises 2238 nucleotides of open reading frame followed by a single termination (TAA) codon and 414 nucleotides of 3′ noncoding sequence (Fig. 1).

The alignments between the deduced amino acid sequences of *BVP1* and *BVP2* and direct internal sequence acquired from the M_r 64,500 to 67,000 polypeptide of the enzyme from the same organism (Rea et al., 1992a; Sarafian et al., 1992) verify that the cDNA clones encode the substrate-binding subunit of the V-PPase. The deduced amino acid sequences of both *BVP1* and *BVP2* show complete identity with all four segments of microsequence over a total span of 66 amino acid residues except for two nonconservative (Ser→Ala and Gln→Ser) substitutions at positions 560 and 562 in the deduced sequence of *BVP2* (Fig. 2).

The differences between the sequences of *BVP1* and *BVP2* are too numerous and widely distributed to have arisen from cloning artifacts and/or individual variation between clones. The coding sequences of *BVP1* and *BVP2* are 79% identical at the nucleotide level and their respective 3′ and 5′ noncoding sequences exhibit less than 28 and 53% identity, respectively. By the same token, the deduced sequences of the polypeptides encoded by *BVP1* and *BVP2* show 89% identity and have computed masses of 80,548 and 79,966 D, respectively, and estimated isoelectric points of 4.84 and 5.05, respectively.

The results of northern analyses demonstrate that the two cDNA species do not originate from alternate splicing of the coding sequence of one transcript with the noncoding sequence of another and further exclude cloning artifacts. When poly(A)$^+$ RNA is isolated from leaves and roots of 7-d-old plants of *Beta*, size fractionated, and hybridized with ^{32}P-labeled riboprobes transcribed from the open reading frame of *BVP1*, to generate a nonspecific probe, or the 3′ noncoding sequences of *BVP1* or *BVP2*, to generate gene-specific probes (Fig. 3), a single hybridizing band migrating at 2.8 kb is obtained in all cases (Fig. 4A). Since the 3′ *BVP1* riboprobe does not hybridize with in vitro transcribed *BVP2* message and vice versa (data not shown), three conclusions follow: (a) The lengths of the cDNA inserts of pBVP1 and pBVP2 and the mature *Beta* transcripts coincide. (b) Both *BVP1* and *BVP2* are expressed endogenously. (c) Neither of the 3′ noncoding sequences are likely to be derived from non-V-PPase-associated transcripts through cloning artifacts or alternate splicing, since the divergent 3′ noncoding sequences of *BVP1* and *BVP2* each hybridize with a 2.8-kb mRNA species, as does the nonspecific coding sequence probe.

In agreement with the results of the sequence and northern analyses, Southern blots of *Beta* genomic DNA probed with the 2.1-kb *Hin*dIII fragment of *BVP1* (Fig. 3) imply that the substrate-binding subunit of the V-PPase is encoded by two genes. Of the three restriction enzymes chosen for the genomic digestions, *Pvu*II has one restriction site within each of the *Hin*dIII fragments of *BVP1* and *BVP2*, *Nco*I has no restriction sites in *BVP1* and one in *BVP2*, and *Hin*dIII has no

A. Coding Sequences

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1     ATGGGTGCAGCTCTTCTTCCAGATCTCAT-AACAGAGAT-TATCATTCCTGTATGTGCTGTAATTGGA-ATTGCTTTCTCTCTCT-TGCAATGGTA-C 98
      ...-----.--.-..----.....-.GC........C.T.-.....G..G..C..C..C..C...T..A--....-..G.-.A.-.......TT.

99    ATCGTT-CTCAGGGTCAAGCTTTCCCCTGATTCTACCCG-CAATAATAAC--AA---C-AAGAATGGCTTTTCTGAT-AGTTTGATTGAAGAAGAAGA 196
      T.-...T..A.A.T.A...-.....A....C-.G..A..T...C...GG.GG..GTG.T..A.........AA....T.C..-..............

197   AGGTCTTAATGATCAAAGTGTTGTTGCTAAATGTGCTGAAATTCAGAATGCTATTTCTGAAGGAGCAACTTCCTTCCTTTTCACCGAGTACCAGTATG 294
      ....G.......C....AC..C..C................C..A..C..C..........T.....A.....T..G.....G..A.........

295   TTGGTATCTTTATG-GTTGCTTTTGCTGTGTTGATATTC--CTTTTCCTCGGATCTGTGGAGGGCTTCAGCACAAGTA-GCCAGGA--ATGTACCTAT 329
      .G..GG.G..C...T..-..A........CC.C..C...GT.....--.A..........A.....T..T...-.A.A....--.CC...C..-.-.

330   GACAAAACCAGGA-G--GTGCAAGCCTGCTCTTG-C--CA-CTGCTATCTTCAGCACAGTGGC-CTT-CTTGCTTGGCGCTATCACTTCT-TTGGGT- 490
      -...GCC..TT..A.AA......A.....A-...G.TA..G..-..-.-........C.--.T...T.........T...........G...--.C

491   TCTGGTTTCTTCGGGATGAAGATTGCCACAT-ACGCAAATGCCCGAACAACACT-AGAGGCTAGAAAGGGTGTCGGCAAAGCATTCATTGTAGCATTC 588
      .........C.A..A..........T..-.G.T........AA.......-..T...A..............T..G..G..T......A.T..C...

589   AGGTCTGGAGCTGTCATGGGATTCCTACTTGCTGCAAATGGTCTTTTGGTGCTTTACATTACTATCC-TTCT-CTTCAAGATTTACTATGGTGATGAC 686
      ..A.....T.....T.....T.....C.....A..C........A.....C.........G.C..TAA..-.G..-....C.G..T..........T

687   TGGGAAGGTCTGTTTGAGGCTATAACTGGTTATGGTCTTGGAGGATCATCCATGGCCCTTTTCGGTAGAGTTGCTGGAGGTATTTACACAAAAGCTGC 784
      ...........T..........T.................T..T..T..G.......................GG........C..T..T..G.....

785   CGATGTGGGTGCTGATCTTGTCGGTAAGGTTGAAAGAGA-CATCCCTGAGGATGACCCCAGAAATCCAGCTGTTATTGCTGACAATGTCGGCGACAAT 882
      T.....A...............A..A............-..A......A........T..A...............G.................T......

883   GTTGGGGATATCGCTGGGTATGGGGTTCTGATCTTTTTGGATAGCT-ACGCTGA-GTCGTCCTGTGCTGCTCTTGTTGTTCGA-TCCATTTCCTCATT 980
      .....T.....T......-.....-...................T.--..T.T.....A.-.A..A........A.........-.CC.....A.....C..

981   CGGAATTTC--CCATGATTTGACGGCAATGATGT-ACCCATTG-TTGGTTAGCTC---GGTTGGTATTATTGTTTGCTTGATCACAAC-CTTATTTGC 1078
      ...T..--.AA...C..G..C..T..C...-...T.T..G..AC..A-.C..T..CAT..--..-..C..A.........G.......T..C-.....

1079  AACCGATTTCTTCGAGATCAAGGCTGTTAAGGAGATTGAGCCTGCACTCAAGAAGCAGCTAATCATCTCCACTGCTCTTATGACTGTCGGAGTTGCAG 1176
      T..T........T..A..T..A.....C..A..A.........A.....G........A..C..T..T..A....T.........C..G...A.....A

1177  TTATTTCTTGGATTGCTCTTCC-TACTTCATTT-ACCATTTTTGACTTCGG-ATCTCAGAAGGAGGT-G-C-AGAACTGGCAATTGTTTTTATGTGT- 1274
      .C..CA.............C..A.C..-.-...C.........CA.T..T..C.-.A..-..--....C.T.C.C..T......C....CC.......A

1275  TGCTGTTGGG-TTGTGGGCTGGGCTCATCATCGGATTTGTGACTGAATACTACACTAGCAATGCATACAGCCCTGTGCAAGATGTTGCTGATTCTTGC 1372
      ..-......TC..-.........A........T..T.....A..A..G........C..T..........T........................C...

1373  CGAACTGGAGCTGCCACAAATGTTATTTTTGGCCTGGCCTTGGGTTACAAATCAGTCATTATTCCTATTTTTGCCATTGCTATCAGCAT-TTTCGTCA 1470
      ..G..A.........................T..A.................T.....C.....C..C.....T..A...G.......C...-....

1471  GTTTTAGCTTTGCAGCTATGTATGGTATTGCTATGGCTGCTCTTGGTATGCTGAGCACCATTGCCACTGGAT-TGGCTATTGATGCATATGGCCCTAT 1568
      ....C..T.....T..C.........G.....G.T.....C.....A.......................-.C.C..C........T.....T..A..

1569  CAGTGATAATGCTGGAGGCATTGCTGAGATGGCTGGTATGAGCCACAGAATCCGTGAGAGAACTGATGCCCTTGATGCTGCTGGAAACACAACCGCTG 1666
      ......C..C........T..............A..G.........C....T...........C...............A..A..G........T....

1667  CTATTGGAAAGGGTTTTGCAATCGGTTCTGCAGCTCTT-GTTTCTCTT-GCTCTCTTTGGTGCTTTTGTAAGCCGTGCATCCATC-CAAACTGTGGAT 1764
      .C.....G.....A.....T..A..A.........-..A..C..-...A.....G........C.....C..T..C...G.T..TT...C.-..T...

1765  GTGTTGACCCCGAAAGTATTCATTGGTCTCATTGTGGGAGCCATGCTTCCATACTGGTTCTCTGCCATGACAATGAAGAGTGTGGGAAGTGCAGCT-T 1862
      ..T.....T..T.....T.....C...T.G.....T..T...........T..............T.....T...............T.....T...C.

1863  TGAAAATGGTTGAAGAGGTCCCGAAG-CAATTCAACACCATCCCTGG-CTTGCTGGAAGGCACTGCCAAACCCGACTATGCTACCTGTGTCAAGATCT 1960
      .-..G.....G..G.......-.C..A..G.....T.......G..T..-.A.......A........G..T..T.....C..T.....T..A....

1961  CCACTGATGCTTCCATCAAGGAGATGATCCCCCCAGGTGCTCTTG-TCATGCTCACACCATTGATTGTTGGAACCTTCTTTGGTGTCGAAACTCTGTC 2058
      ..........A..T................A..T.....G-...G..........C...C.C.........T............T..G..C..A..

2059  TGGCGTTCTTGCTGGTTCTCTTGTGTCTGGTGTACAGATTGCTATTTCTGCATCCAACACTGGTGGTGCTTGGGACAATGCCAAGAAGTACATTGAGG 2156
      ...T.....A.....A..C.....T..C..A..C.....A..A..A.....C..................A.........................C....

2157  CTGGTGCTTCAGAGCATGCAAGGACACTTGGTCCCAAGGGATCAGATGC-ACACAAGGCAGCTGTGATCGGTGACACCATCGGTGACCCACTTAAGGA 2254
      ........................GT.....A..A.....G..T..-..C............A..C..T.....T.....T...........C.AGGA

2255  CACATCAGGACCATCACTCAACATTCTAATCAAGCTTATGGCTGTCGAGTCACTAGTGTTCGCCCCCTTCTTCGCCACCCACGGTGGCTTGCTCTTCA 2352
      ...C.....C..T...T.G......T.G.....A........C..T.....T..C..T.....T..A.....T..A..T..T.....T...T.G....

2353  AG-TACCTC                                                                                          2361
      .AC..TT.
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B. Noncoding Sequences

I. 5' Sequences

BVP1:

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-57    TTTCTCTCT CCTTTATCTT ATTCTTCTTC AATTTTCTTC TCCCATTTTC AAAAATC -1
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BVP2:

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-200   TGACTTCTGA GCTTAACCTT GGGTGGCAAA TTCATCCATT AGTTTGTCTC AGCGCAGGTT TCTGTCTCTC TTTCAATTGC CAGCACTACG -111
-110   CACAACTACA CACTCAAATT GTGTTGTTGT CTTAACTGTA TTTTCTCTGT TGCCGGAATT TCGGCGTTTC TTAGGGTTCC GGCGTAAAGT -21
-20    TAGGGTTCCG GTGAAGAAAA                                                                              -1
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II. 3' Sequences

BVP1:

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2296   TAATGAATGA TCGGCGCAAA ATCAGAAGGC GACAGAGGGA GGAATTCGCG GTTTCTTCTC CTCATTTTAT CGCCTACAAA TCGGGCAAGT 2385
2386   TTTAAATTTT ATCGCACAAT TTTTGAATGT CGTTAGATGA CAACTACAAG GCTGGAGGGG CTAAAACTTC TACATGATGA TGATGATAAT 2275
2476   TTGGAAGCAA GTCTTGTGAA AAATAGAGTT ATATGGTCAA CATTATTCTT TTCTTTTTTG TTCCTTTTAT TGTAAGATCG GGATTTGTAG 2567
2566   TAATCATTTT GCAAACCTCT TTTGTTAGGT ATAACTCATT TTCTATTTTA GTCCTTCAGA AATTGTATGC AGTTGCCTTT TTATTTTCTA 2655
2656   AAAAGAGAAC CTGTTCTTGA GCATCTGTTG TAAACGCATA ATATTCTCTG TACTTTCTTG GAATTTATCT CATTTTGCAG ATTGGATCTA 2745
2746   AAAAAAAAAA AAAAAAAA                                                                                2763
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BVP2:

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2284   TAAGAGGATG CCAAATGTGA ATTCAACAGG AAGGTCAATT TTTGAGCTTC TCTTCAGTCT TTATGGGCGA ATGCAGAATG CGCATTAAGC 2373
2374   AGACATCCTG TTGTACTCAT TTCTATCATG GCTCAGTTTT TGCCCCAATT TTTTTGGGTT TTGTCTGTTA GTTTTGGTAG AGGTAAGGAT 2463
2464   GATTGTTGTC ACTTATGATG ATTGTAGGAA TGATTATGCA TTTCGAGTTT AGAGGAAAGA AGATCTGCGG TTAGTTGTTA CCTTTGTTGT 2553
2554   AGGAAAGGTT GATAGATCAA CCACCAATGT TGTTAGATCG GAATGAGCAA AATGTTGCCA TCTTGTTGAG GGTATAAATT TTGTTGGCTT 2643
2644   CTTTGTAACA TCGATGATTT GATGTAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAA                                     2700
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Figure 1. (Legend appears on facing page.)

A.

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BVP1:   MetGlyAlaAlaLeuLeuProAspLeuIleThrGluIleIleIleProValCysAlaValIleGlyIleAlaPheSerLeuLeuGlnTrpTyr
Vigna:  ---GlyAlaAlaIleLeuProAspLeuGlyThrGluIleLeuIleProVal???AlaValIleThrIleAlaPheAlaLeuPheGlnTrpLeu

BVP2:   ---------MetIleSer---AspLeuAlaThrGluIlePheIleProValCysAlaValIleGlyTyrIleSerCysIle------GlnTrpPheLeu
Vigna:  ---GlyAlaAlaIleLeuProAspLeuGlyThrGluIleLeuIleProVal???AlaValIleThr---IleAlaPheAlaLeuPheGlnTrp---Leu
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B.

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1    MGAALLPDLITEIIIPVCAVIG-IAFSLLQWYIVLRVKLSPDSTRNNNN----KNGFSDSLIEEEEGLNDQSVVAKCAEIQNAIS 80
     MIS----DLATEIFIPVCAVIGYI--SCYQWFLVSKVKVSTD--RHVNNGGSAKNGFNDYLIEEEEGVNDQNVVAKCAEIQNAIS 77

86   EGATSFLFTEYQYVGIFMVAFAVLIFLFLGSVEGFSTSSQECTYD--KTRRCKPALATAIFSTVAFLLGAITSLGSGFFGMKIAT 163
     EGATSFLFTEYQYVGVFMCAFAVLIFVFLGSVEGFSTESQPCTYSPLKK--CKPALATALFSTVSFLLGAITSVVSGFLGMKIAT 160

171  YANARTTLEARKGVGKAFIVAFRSGAVMGFLLAANGLLVLYITI-LLFKIYYGDDWEGLFEAITGYGLGGSSMALFGRVAGGIYT 247
     DANARTTLEARKGVGKAFIIAFRSGAVMGFLLAANGLLVLYIAINLL-KLYYGDDWEGLFEAITGYGLGGSSMALFGRVGGGIYT 244

256  KAADVGADLVGKVERDIPEDDPRNPAVIADNVGDNVGDIAGYGV-LIFLDSYAESSCAALVVRSISSFGISHDLTAMMYPLLVSS 331
     KAADVGADLVGKVERNIPEDDPRNPAVIADNVGDNVGDIAGMGSDL-FG-SYAEASCAALVVASISSFGINHEFTAMCYPLLISS 328

341  VGIIVCLITTLFATDFFEIKAVKEIEPALKKQLIISTALMTVGVAVISWIALPTSFTIFDFGSQKEVQNWQLFLCVAVGLWAGLI 416
     MGIIVCLVTTLFATDFFEIKAVKEIEPALKKQLIISTVLMTVGIAIITWIALPSSFTIFNFGTQKVVHNWQLFLCVCVGLWAGLI 413

426  IGFVTEYYTSNAYSPVQDVADSCRTGAATNVIFGLALGYKSVIIPIFAIAISIFVSFSFAAMYGIAMAALGMLSTIATGLAIDAY 501
     IGFVTEYYTSNAYSPVQDVADSCRTGAATNVIFGLALGYKSVIIPIFAIAVSIFVSFSFAAMYGVAVAALGMLSTIATGLAIDAY 498

511  GPISDNAGGIAEMAGMSHRIRERTDALDAAGNTTAAIGKGFAIGSAALVSLALFGAFVSRASIQTVDVLTPKVFIGLIVGAMLPY 586
     GPISDNAGGIAEMAGMSHRIRERTDALDAAGNTTAAIGKGFAIGSAALVSLALFGAFVSRAAISTVDVLTPKVFIGLIVGAMLPY 583

596  WFSAMTMKSVGSAALKMVEEVPKQFNTIPGLLEGTAKPDYATCVKISTDASIKEMIPPGALVMLTPLIVGTFFGVETLSGVLAGS 671
     WFSAMTMKSVGSAALKMVEEVRRQFNTIPGLMEGTAKPDYATCVKISTDASIKEMIPPGALVMLTPLIVGIFFGVETLSGVLAGS 657

681  LVSGVQIAISASNTGGAWDNAKKYIEAGASEHARTLGPKGSDAHKAAVIGDTIGDPLKDTSGPSLNILIKLMAVESLVFAPFFAT 756
     LVSGVQIAISASNTGGAWDNAKKYIEAGASEHARSLGPKGSEPHKAAVIGDTIGDPLKDTSGPSLNILIKLMAVESLVFAPFFAT 751

766  HGGLLFKYL                                                                             766
     HGGLLFK-LF                                                                            761
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Figure 2. Deduced amino acid sequences of polypeptides encoded by BVP1 and BVP2. A, Alignment of direct N-terminal sequence of the substrate-binding subunit of V-PPase from *V. radiata* (Maeshima and Yoshida, 1989) and deduced N-terminal sequences of BVP1 and BVP2. B, Alignment of total deduced sequences of BVP1 and BVP2. The underlined amino acid residues align with direct sequence acquired from the substrate-binding subunit of the V-PPase purified from *Beta* (Sarafian et al., 1992; Rea et al., 1992b). Dashes denote gaps introduced to maximize alignment; colons denote conservative substitutions; lightface numbers (left) denote residue positions including gaps; boldface numbers (right) denote residue positions excluding gaps.

further restriction sites in either the *Hin*dIII fragment of *BVP1* or the entire sequence of *BVP2* (Fig. 3). Providing that the region or regions of the genome corresponding to the 2.1-kb *Hin*dIII fragment of *BVP1* or the corresponding sequence of *BVP2* are devoid of introns containing identical restriction sites, two bands from the *Hin*dIII genomic digest, four bands from the *Pvu*II digest, and three bands from the *Nco*I digest would be expected if *BVP1* and *BVP2* are each encoded by a single gene. This is what is found. Genomic DNA digested with *Pvu*II (or *Nco*I) and probed with the 2.1-kb fragment yields intensely hybridizing bands at 3.3, 1.5, and 0.6 kb (or 3.7, 1.9 and 1.0 kb) (Fig. 4B). The *Hin*dIII digest, by contrast, contains only two intense bands at approximately 3.1 and 2.6 kb (Fig. 4B).

DISCUSSION

The data presented indicate that the substrate-binding subunit of the V-PPase of *Beta* is specified by two genes,

Figure 1. (on facing page). Nucleotide sequences of cDNA inserts of pBVP1 and pBVP2. A, Alignment of open reading frames of BVP1 (top sequence) and BVP2 (bottom sequence). B, 5′ and 3′ noncoding sequences of BVP1 and BVP2. Dots denote nucleotides identical between BVP1 and BVP2; dashes denote gaps introduced to maximize alignment. The alignments were achieved using the maximum-matching algorithm of DNASIS, version 2.0 (Hitachi Software Engineering Co., Ltd.).

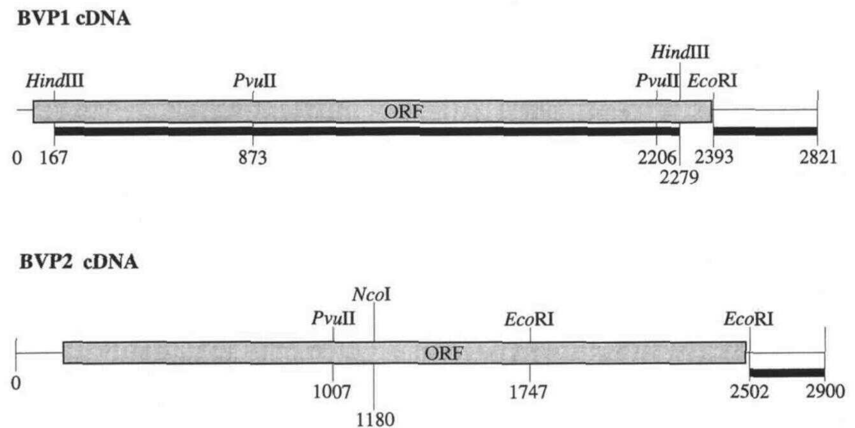

Figure 3. Restriction maps of the cDNA inserts of pBVP1 and pBVP2. The positions of the relevant restriction sites are indicated. The 5′ and 3′ ends of the inserts demark the *Xba*I and *Xho*I sites, respectively. Stippled blocks, Open reading frame (ORF); solid blocks, sequences encompassed by the riboprobes used for the northern analyses shown in Figure 4A.

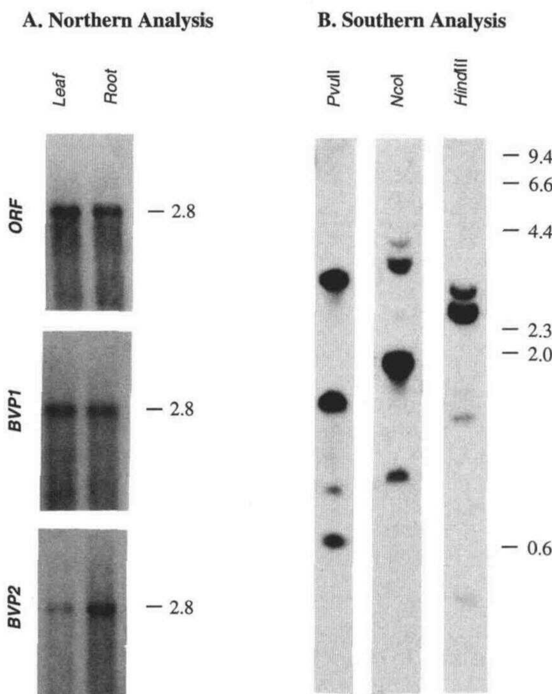

Figure 4. A, Northern blot analysis of *Beta* V-PPase. Five-microgram aliquots of poly(A)$^+$ RNA isolated from roots and shoots of 1-month-old plants were electrophoresed, blotted, and hybridized with ^{32}P-labeled riboprobes transcribed from the *Hin*dIII fragment (open reading frame [ORF]) of *BVP1*, *Eco*RI/*Xho*I fragment (3′ noncoding sequence) of *BVP1* or *Eco*RI/*Xho*I fragment (3′ noncoding sequence) of *BVP2* (Fig. 3). B, Genomic Southern blot analysis of *Beta* V-PPase. Genomic DNA was isolated from 7-d-old seedlings of *B. vulgaris*. After digestion with a restriction enzyme, samples of the DNA (4 μg) were electrophoresed on 0.8% agarose gels and transferred to nylon membrane filters for hybridization with the 2.1-kb ^{32}P-labeled *Hin*dIII fragment of *BVP1* (Fig. 3). Genomic DNA was digested with *Pvu*II (lane 1), *Nco*I (lane 2), or *Hin*dIII (lane 3).

both of which are expressed in the intact plant. The two genes do not appear to be alleles since the corresponding cDNAs contain divergent 5′ and 3′ noncoding sequences. Nor are they likely to encode sequence-related subunits of a heteromultimeric complex. The results of both protein chemical and molecular studies suggest that the substrate-binding subunit is the sole polypeptide constituting the functional V-PPase complex. This subunit alone co-purifies with V-PPase activity during detergent solubilization and chromatography (Britten et al., 1989; Maeshima and Yoshida, 1989; Rea et al., 1992a) and is the only component of vacuolar membrane vesicles to undergo Mg_2PPi-protectable, free PPi-potentiated covalent modification by [^{14}C]*N*-ethylmaleimide (Britten et al., 1989; Rea et al., 1992a). Moreover, heterologous expression of the one cDNA species (AVP) encoding the substrate-binding subunit of the V-PPase from *Arabidopsis* in the yeast *Saccharomyces cerevisiae* results in the production of vacuolarly localized enzyme that is indistinguishable from the endogenous plant enzyme with respect to PPi hydrolysis, H^+-translocation, activation by K^+, and selective inhibition by Ca^{2+} and 1,1-diphosphonates (Kim et al., 1994). Therefore, we infer that additional subunits need not be invoked to account for V-PPase function in *Arabidopsis*, which, in turn, implies that *BVP1* and *BVP2* encode variants, possibly isoforms, of the same subunit in *Beta*.

Two further points are noteworthy concerning the deduced sequences of the translation products of *BVP1* and *BVP2*. First, as described before for the cDNAs encoding the V-PPase from *Arabidopsis* (Sarafian et al., 1992) and *Hordeum* (Tanaka et al., 1993), the open reading frames of *BVP1* and *BVP2* encode polypeptides with computed masses of approximately 80 kD, but the corresponding subunit(s) from *Beta* has an M_r of 64,500 to 67,000 (Britten et al., 1989; Sarafian and Poole, 1989; Rea et al., 1992a). Although the possibility of posttranslational proteolysis of the polypeptides concerned cannot be completely discounted, two considerations make this seem improbable: (a) The direct sequence acquired from the M_r 64,500 to 67,000 and 66,000 to 73,000 substrate-binding subunits of *Beta* and *Vigna*, respectively, include both the N terminus and sequences within 24 amino acid residues of the C terminus (Fig. 2). Although C-terminal proteolysis

is not excluded by the direct sequence data, such a process alone would not be able to account for the apparent mass shifts observed. (b) The endogenous V-PPase of *Arabidopsis* is constituted of a single polypeptide with an apparent M_r of 66,800 (Rea et al., 1992a), and the corresponding immunoreactive polypeptide found after expression of AVP in *Saccharomyces* has an identical M_r (Kim et al., 1994). If posttranslational modification were operative, it would have to be by identical mechanisms in both yeast and plants and, in the former case, would necessitate sequence-specific modification of a protein that is otherwise not found endogenously. It is therefore suggested that, in common with a number of other hydrophobic membrane proteins, the substrate-binding subunit of the V-PPase migrates anomalously on SDS gels. The binding of nonsaturating amounts of SDS by membrane proteins is a common phenomenon and is frequently accompanied by shifts in M_r as a result of irregularities in the shape of the SDS-protein complex, exposure of charged amino acid residues, or both (Branden and Tooze, 1991).

Second, one of the motifs previously proposed to participate in substrate (Mg^{2+} and/or PPi) binding is not conserved between the two isoforms from *Beta* and the corresponding polypeptide from *Arabidopsis*. X-ray crystallographic and site-directed mutagenesis studies of the soluble PPase from *S. cerevisiae* have disclosed 17 residues that are thought to participate directly in catalysis, and 11 to 16 of these residues (depending on alignment procedure) are conserved in all sequenced soluble PPases (Cooperman et al., 1992). Therefore, it has been considered of potential functional significance that eight of the active-site residues of soluble PPases fall into two configurations, $E(X)_7KXE$ and $D(X)_4DXK(X)_4D$, beginning at positions 48 and 146, respectively, in the sequence of the enzyme from *Saccharomyces* and that variants of these motifs, $D(X)_7KXE$ and $E(X)_4DXK(X)_4D$, with an equivalent spacing and alternation of acidic (D and E) and basic (K) residues are found at positions 257 and 119, respectively, in the deduced sequence of the V-PPase from *Arabidopsis* (Rea et al., 1992b). Notwithstanding the marked overall sequence divergence between the V-PPase and soluble PPases, it has been suggested that they may share convergent motifs related to the need for both classes of enzyme to interact with the same substrates, inhibitors, and activators (MgPPi, Mg_2PPi, Ca^{2+}, Mg^{2+}) (Rea et al., 1992b). The deduced sequences shown in Figure 2, however, contradict this hypothesis, at least in its most restrictive sense. Although the $D(X)_7KXE$ motif at position 257 in the sequence of AVP is conserved in both *BVP1* and *BVP2*, the $E(X)_4DXK(X)_4D$ motif is not. AVP has the sequence EGFSTDNKPCTYD, but the equivalent sequences of *BVP1* and *BVP2* are EGFSTSSQECTYD and EGTSTESQPCTYS, respectively (starting at alignment position 118 in Fig. 2). Of the four charged residues tentatively assigned a role in catalysis in this region of AVP (E^{119}, D^{124}, K^{126}, and D^{131}) only one (E^{119}) is conserved between *BVP1* and *BVP2*. Thus, it is extremely unlikely that these residues directly participate in the substrate hydrolytic cycle of the V-PPase. Any similarities between AVP and the sequences of soluble PPases, at least so far as the $E(X)_4DXK(X)_4D$ motif is concerned, are, therefore, probably coincidental.

It is not known why isoforms of the V-PPase should be found in *Beta*. Northern analyses show that the two isoforms are expressed at similar levels, and a broader range of analyses of seeds that had been allowed to imbibe, whole 7-d-old dark, and light-grown seedlings, leaves, roots, and storage roots of 7-month-old plants, as well as leaves and roots of 1-month-old plants, similarly failed to reveal consistent and significant differences between the steady-state levels of expression of *BVP1* and *BVP2*. Therefore, it is apparent that higher resolution techniques, such as in situ hybridizations (Coen et al., 1990) or β-glucuronidase reporter analyses (Jefferson et al., 1987), or exposure of the tissues concerned to a wider range of growth conditions are needed to determine whether multiple genes for the V-PPase provide isoforms with expression characteristics unique to specific cell types, developmental stages, and/or environmental factors or generate enzyme molecules with modified catalytic and/or regulatory characteristics suited to the particular H^+- and/or K^+-translocation requirements of the tissues, cells, or endomembranes in which the V-PPase is located.

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