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Isolation and characterization of cDNAs encoding vacuolar H^+-pyrophosphatase isoforms from rice (*Oryza sativa* L.)

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Abstract

The vacuolar H^+-pyrophosphatase (V-PPase) is an electrogenic H^+ pump, which was found in the plant vacuolar membrane. Two cDNA clones (OVP1 and OVP2) encoding the V-PPase were isolated from cultured rice (*Oryza sativa* L.) cells and subsequently sequenced. The sequence analysis has revealed that *OVP1* contains 2316 nucleotides of open reading frame (ORF) and 362 nucleotides of the 3′-untranslated region, whereas *OVP2* comprises 2304 nucleotides of ORF and 312 nucleotides of the 3′-untranslated region. The nucleotide sequences of ORF of *OVP1* and *OVP2* are 80.7% identical, and their 5′- and 3′-untranslated regions have 39.4% and 48.4% identity, respectively. The polypeptides encoded by the ORF of *OVP1* and *OVP2* contain 771 and 767 amino acids, respectively, and the sequences of the OVP proteins are very similar to those of other V-PPases, which are shown to have 85–91% homology. Chromosomal mapping by RFLP techniques demonstrates that OVP1 and OVP2 are isoforms encoded by different genes. Both *OVP1* and *OVP2* are mapped on the same chromosome (chromosome 6) to a distance of ca. 90 cM. Northern analysis indicates that the *OVP1* and *OVP2* are also expressed in intact rice plants and *OVP2* shows higher expression in the calli than the roots and shoots, compared to *OVP1*. These results show that at least two genes encoding the V-PPases are present in rice genome and their expressions are probably regulated in a different manner.

Introduction

In plant cells, H^+ pumps play important roles in metabolism, homeostasis and regulation of turgor pressure by establishing transmembrane electrochemical gradients which drive translocations of various solutes such as ions, amino acids and sugars across cellular membranes. The vacuolar membrane of plant cells contains two different H^+ pumps, i.e., vacuolar H^+-ATPase (V-ATPase; EC 3.6.1.3) and H^+-translocating inorganic pyrophosphatase (V-PPase; EC 3.6.1.1) [34, 35, 41]. Although the V-ATPase exists in various acidic intracellular compartments of yeast [13], fungi [3], plants [24] and animals [5, 30], the V-PPase has been found only in plants (vascular plants, algae and moss) [12, 22, 33, 38]. The primary structure of the V-PPase sequence shows no similarity to those of mitochondria PPase and soluble PPase. The inhibitor sensitivity of the V-PPase is also different from that of other H^+ pumps. F_0F_1-, P-, and V-ATPases are selectively inhibited by azide, vanadate, and bafilomycine, respectively [27]. None of these inhibitors inhibit the V-PPase. The V-PPase is specifically inhibited by aminomethylendiphosphonate [45] and 3, 3′, 4′, 5-tetrachlorosalicylamide [12].

Despite the dissimilarity of the primary structure, two putative catalytic sites of the V-PPase were found on the basis of the sequence comparison between the V-PPase of *Arabidopsis thaliana* (*AVP*) and yeast soluble PPase, of which the potential catalytic residues

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers D45383 (*OVP1*) and D45384 (*OVP2*).

had been characterized by X-ray crystallography and site-directed mutagenesis [32]. However, one of the putative catalytic sites is not universal, because the sequences in the corresponding region of AVP are different from those of the V-PPases from the other plant species [16, 20]. The protein chemical studies using the sulfhydryl reagent N-ethylmaleimide (NEM) and the membrane-impermeant cysteine reagent 3-(N-maleimidylpropionyl)biocytin (MPB) demonstrated that Cys-634, a universal residue, is responsible for inactivation by maleimides and is cytosolically disposed [46]. Furthermore, an investigation using site-directed mutagenesis and the yeast expression system showed that Cys-634 was necessary for inhibition of the V-PPase by NEM but not directly concerned with catalysis [15]. This Cys-634 is the only ligand-active residue which has been identified in V-PPase.

It has been known that primary ion translocases usually have several isoforms and are encoded by a multigene family. For instance, there are more than 10 isoforms of the plasma membrane H^+-ATPase in *Arabidopsis* (*AHA1–10*) [39] and at least four cDNA clones encoding the 16 kDa proteolipid subunit of the V-ATPase in oats [42]. In addition, each isoform of the plasma membrane H^+-ATPase is probably expressed in a tissue- and organ-specific manner and different from each other in their biochemical and regulatory characteristics [7, 11, 31]. Moreover, chromosomal mapping indicated that the genes encoding the isoforms of the plasma membrane H^+-ATPase of tomato and potato were different and were located on distinct sites in their chromosomes [9, 10].

To date, several cDNA clones of the V-PPase have been reported from various plants. A single cDNA clone has been isolated from *A. thaliana* (*AVP*) [37] and *Hordeum vulgare* (*HVP*) [43], and two cDNA species of *Beta vulgaris* (*BVP1* and *BVP2*) [16] and three species of *Nicotiana tabacum* (*TVP5*, *TVP9* and *TVP31*) [20] have been cloned. These findings indicate that *Beta* and tobacco have at least two and three V-PPase isoforms, respectively. However, it remains unclear whether V-PPase isoforms are encoded by a multigene family as other primary ion translocases. In this report, we have isolated two cDNA clones encoding the V-PPase from rice and demonstrated that they are isoforms encoded by different genes by the determination of their chromosomal locations. Their expression in rice tissues was also investigated.

Materials and methods

Plant material

Rice (*Oryza sativa* L. cv. Nipponbare) cells [14] cultured for 7 days at 25 °C in liquid media were prepared for extraction of RNA and DNA. Rice plants were hydroponically grown for 10 days at 25 °C for preparation of RNA from shoots and roots.

Preparation of the cDNA probe

Two degenerate oligonucleotides, a 25-mer designated PP1 (5′-GGAAGCTTAT(C)TAT(C)GGIGAT(C)GAT-(C)TGG-3′) and an antisense 28-mer, PP2C (5′-GG AAGCTTGTIGTA(G)TTICCIGCA(G)TC-3′), which contain *Hin*dIII linkers and nucleotide sequences (underlined) corresponding to amino acids 219 to 224 (YYGDDW) and 534 to 540 (DAAGNTT) of the deduced amino acid sequence of AVP [37], were synthesized. Total RNA was extracted from the calli by the guanidinium method [5]. cDNA was synthesized from 1 μg of the total RNA by reverse transcriptase (SuperScript RT, Gibco-BRL), and PCR amplification was subsequently performed using PP1 and PP2C as primers in an automated thermal cycler (Nippon Genetics). The PCR product (1.0 kb) was digested with *Hin*dIII and ligated into pBluescript SK+ (Stratagene). Nucleotide sequences of the inserts were determined by the dideoxynucleotide method [36] using the Dye Primer Cycle Sequencing kit and an automated DNA sequencer 373A (Perkin Elmer).

Construction and screening of the cDNA library

Poly(A)$^+$ RNA was isolated by Oligotex-dT30 (Takara) from the total RNA of rice calli. A cDNA library was constructed using a ZAP-cDNA synthesis kit (Stratagene). After cDNA was synthesized from 5 μg of the poly(A)$^+$ RNA with *Xho*I-poly(dT) primer and ligated to *Eco*RI adapters, it was size-fractionated at greater than ca. 2 kb by agarose electrophoresis and ligated into uni-ZAP XR vector arms. The ligated cDNA was packaged using Gigapack II gold packaging extracts (Stratagene) and amplified once. Therefore, a library whose titer was 1×10^{10} plaque-forming units/ml was obtained. The cDNA library was screened using the PCR product which contains a sequence homologous to that of AVP as a probe. About 3×10^5 phage were plated, transferred onto nylon membranes (Hybond N+, Amersham) and hybridized using

an ECL random prime labelling system (Amersham). Hybridization was carried out overnight at 60 °C in 5× SSC with 0.1% SDS, 0.5% blocking agent, 100 μg/ml herring sperm DNA and 5 ng/ml probe. Filters were washed twice for 15 min in 1× SSC, 0.1% SDS at 60 °C and then for 15 min in 0.5× SSC, 0.1% SDS at 60 °C. To detect signals, the filters were incubated with anti-fluorescein horseradish peroxidase conjugate and washed again. After secondary and tertiary screening under the same conditions, insert-containing plasmids were isolated by *in vivo* excision from hybridizing phages. Restriction and partial sequence analysis showed that the obtained clones could be divided into four different classes. Both strands of the longest inserts from each of the two major classes, named OVP1 and OVP2 (for *Oryza sativa* vacuolar H^+-pyrophosphatase), were sequenced using an automated DNA sequencer. The DNA sequences were analyzed using a GENETYX software package version 7.0 (Software Development). Hydropathy profiles of the deduced amino acid sequences of the V-PPases were also analyzed using the GENETYX software. Transmembrane segments of the polypeptides were predicted using PSORT programmed by K. Nakai (Osaka University, Japan) [25].

Northern and Southern hybridizations

Total RNA was isolated from calli, shoots and roots as described above. After fractionation by electrophoresis on 1.2% agarose gel containing 0.66 M formaldehyde, the RNA was blotted onto a nylon membrane. Genomic DNA isolated from calli [2] was digested with the indicated restriction enzymes (Takara), fractionated on 1% agarose gel and blotted onto a nylon membrane. For gene-specific probes, the 3′-untranslated regions of *OVP1* (2376 to 2678) and *OVP2* (2306 to 2616) subcloned into pBluescript were isolated and labelled with [^{32}P]dCTP (Amersham) by random priming (Multiprime DNA labelling system, Amersham). Both northern and Southern hybridizations were performed in a buffer containing 5× SSPE, 0.1% SDS, 5× Denhardt's solution, 50% formamide, 100 μg/ml herring sperm DNA and ^{32}P-labelled DNA probe for 12 h at 42 °C. Filters were washed twice for 10 min at 42 °C in 2× SSC, 0.1% SDS and then for 30 min at 42 °C in 1× SSC, 0.1% SDS for northern and Southern hybridization. In the case of Southern hybridization, the filters were washed once more for 30 min at 42 °C in 0.1× SSC, 0.1% SDS.

RFLP mapping

RFLP mapping was carried out in an F_2 population of *O. sativa* L. cv. Nipponbare × cv. Kasalath consisting of 186 individuals that had been scored for over 1300 DNA markers. RFLP between both parents and segregation patterns in F_2 plants were detected by hybridization to blots of the genomic DNA and linkage analysis was performed by MAPMAKER/EXP 3.0 as described [19]. This experiment was carried out in the Rice Genome Research Program (National Institute of Agrobiological Resources/Institute of Society for Techno-Innovation of Agriculture, Forestry and Fisheries, Japan).

Results

Cloning and sequencing

Two primers were synthesized from the consensus amino acid sequences of AVP [37] and were used in PCRs with cDNA reverse-transcribed from mRNA of rice calli. The nucleotide sequence of 1.0 kb PCR-amplified DNA was homologous to those of *AVP*. About 3×10^5 plaques of a rice callus cDNA library were screened using the PCR-amplified DNA as a probe. About 100 hybridizing clones were identified, of which 20 clones were further characterized. Restriction patterns and partial sequence analyses showed that the V-PPase-like clones could be classified into four classes. Two of these classes, classes I and II, contained the clones encoding full-length cDNA of the V-PPase, but all clones which belong to the other two classes, classes III and IV, lacked their 5′-terminal regions. We then analyzed further the clones of class I (named OVP1) and class II (named OVP2). The longest clones from each of classes I and II, represented by four and eight clones, respectively, were sequenced completely. There are two ATG codons in the sequence of the 5′-terminal region of *OVP1* (Fig. 1). The sequence around the first ATG codon (GTACatgAA) in *OVP1* does not match the consensus sequence (AACAaugGC) for plant translational initiation sites [21], but the surrounding sequence of the second ATG codon (AGCAatgGC) which is located at 33-nt downstream from the first ATG is identical to the consensus sequence except for guanine(G) in position −3. It was found that G was also present in position −3 of plant mRNAs. Hence, the second ATG codon is identified as the translational initiation site

for *OVP1*. Because the sequence around the first ATG codon of *OVP2* (CGCCatgGC) (Fig. 1) shares a common sequence with the consensus sequence, it is recognized as the translational initiation site. *OVP1* contains 2316 nucleotides of open reading frame (ORF) and 362 nucleotides of the 3′-untranslated region, whereas *OVP2* comprises 2304 nucleotides of ORF and 312 nucleotides of the 3′-untranslated region. The ORFs of *OVP1* and *OVP2* are 80.7% identical and their 5′- and 3′-untranslated regions show 39.4% and 48.4% identity, respectively. This result shows that *OVP1* and *OVP2* are different genes encoding closely related proteins.

Sequence comparison

The polypeptides encoded by the ORFs of *OVP1* and *OVP2* contain 771 and 767 amino acids and have calculated masses of 80607 and 80044, respectively (Fig. 2). The lengths of these polypeptides from the deduced amino acid sequences are similar to those of AVP (770 amino acids) [37], HVP (761 amino acids) [43], BVP1 (765 amino acids), BVP2 (761 amino acids) [16], TVP5 (764 amino acids), TVP9 (765 amino acids) and TVP31 (766 amino acids) [20]. The deduced amino acid sequences of OVP1 and OVP2 are also analogous to those of AVP, BVP1, BVP2, HVP and TVP, which have 85–91% homologies (Table 1). These similarities and the lengths of the transcripts from the result of northern analysis (Fig. 5) indicate that the isolated *OVP1* and *OVP2* genes fully encode the amino acid sequence for the V-PPases. Alignment of the deduced amino acid sequences from the V-PPase genes indicates that the sequences of the N-terminal region (about 50 amino acid residues downstream from the translational initiation methionine) are completely different, although those of the internal and the C-terminal regions are highly conserved (Fig. 2). Hydrophathy profiles predicted from the amino acid sequences of the V-PPases demonstrate that they are hydrophobic integral membrane proteins, and 14 putative transmembrane segments are detected by the PSORT program based on Klein's method [17] (Fig. 2).

Southern analysis and RFLP mapping

The results of Southern analysis of rice genomic DNA probed with the 3′-untranslated regions indicate that each of *OVP1* and *OVP2* exists as a single gene in the rice genome and the chromosomal locations of the two genes are different (Fig. 3). In order to investigate the chromosomal locations of *OVP1* and *OVP2* in detail, we used RFLP techniques. Consequently, they were mapped to the same chromosome, i.e., chromosome 6. The location of each gene in chromosome 6 has been indicated with their position relative to previously mapped markers by Kurata *et al.* [19] (Fig. 4). *OVP1* is mapped to the identical locus to *C554*; however, the nucleotide sequences between *OVP1* and *C554* are distinct. They are different genes and could not be separated by resolution of the system used in this investigation. *OVP2* is located at distances of 0.3 cM from *C226A* and 1.6 cM from *G165A*. Thus, the distance between *OVP1* and *OVP2* is ca. 90 cM. This demonstrates that OVP1 and OVP2 are the V-PPase isoforms which are encoded by different genes and do not originate from alternate splicing of the same transcript.

Northern analysis

RNA was isolated from 10-day-old rice seedlings and 7-day-old calli. To analyze the expression patterns of the individual genes, the 3′-untranslated regions of *OVP1* and *OVP2* were used as gene-specific probes. A single hybridizing band with an approximate length of 2.8 kb was detected in all cases (Fig. 5). The lengths of *OVP1* and *OVP2* are consistent with the size of the mature V-PPase transcript. The cDNA library used for screening was constructed from the poly(A)$^+$ RNA of rice cultured cells. The mRNAs of both OVP1 and OVP2 were found not only in calli but also in shoot and root tissues. Therefore, the expressions of these genes are not specific to calli and are expressed in intact rice cells. Both genes are more highly expressed in roots than in shoots. *OVP2*, however, showed higher expression in the calli, compared to *OVP1*. This reflects the difference in the number of the isolated cDNA clones between OVP1 and OVP2.

Discussion

In this study, we newly isolated two cDNA clones (*OVP1* and *OVP2*) encoding the V-PPase of rice and demonstrated that OVP1 and OVP2 were isoforms encoded by the different genes. Before the cloning of the cDNA, immunoblot analysis using antiserum raised against the V-PPase from *Vigna radiata* (generously provided by M. Maeshima, Nagoya University, Japan) [23] showed that the V-PPase having an analogous structure existed in the tonoplast of rice (data not shown). The results of isolation of the cDNA

OVP1

-64
gtcgaatcctcctccgagaagaggagagtacatgaatcctagcgcgaggatctcgcaggt

agcaATGGCGGCGATACTGCCGGACCTGGCGACGCAGGTGCTGGTCCCGGCGGCGGCCGTG
M A A I L P D L A T Q V L V P A A A V

OVP2

-94
gagacgcgtcacctcctccccgccgccgccgccgccgcctcgcctccgttgcaaccgtag

tagggttagggttttcgtggtgggcgccgccgccATGGCGATCCTCTCGGCGCTCGGGACC
M A I L S A L G T

Consensus sequence for plant translational initiation site

-4	-3	-2	-1	+1	+2	+3	+4	+5
a	a	c	a	a	t	g	g	c
(c)	(g)	(a)	(c)					

Fig. 1. Nucleotide sequences of the 5′-terminal regions of *OVP1* and *OVP2*. The first ATG codon is underlined and the second is double-underlined. The predicted amino acid sequences are shown in one-letter code below the first base of each corresponding codon. The consensus sequence for plant translational initiation site is reported by Lütcke *et al.* [21]. Nucleotides in parenthesis at each position are the second most frequent next to those of the consensus sequence.

Table 1. Percentage of amino acid homology among predicted V-PPase proteins of *O. sativa* (OVP1 and 2), *A. thaliana* (AVP) [37], *B. vulgaris* (BVP1 and 2) [16], *H. vulgare* (HVP) [43] and *N. tabacum* (TVP5, 9 and 31) [20]. Data are calculated by the Homology program from the GENETYX software package.

	OVP1	OVP2	AVP	BVP1	BVP2	HVP	TVP5	TVP9	TVP31
OVP1	100	87.8	86.6	84.8	87.0	85.6	85.7	88.4	86.9
OVP2		100	84.9	85.5	87.7	91.4	86.3	87.9	86.7
AVP			100	87.1	89.0	85.8	88.7	89.7	87.7
BVP1				100	88.1	87.0	87.7	89.4	88.7
BVP2					100	87.4	89.7	91.1	90.2
HVP						100	85.2	87.8	87.8
TVP5							100	93.6	88.3
TVP9								100	91.4
TVP31									100

clones and analysis of the nucleotide sequences showed that the deduced amino acid sequences of OVP1 and OVP2 were very similar to those of the other cloned V-PPases with 85–91% homology (Table 1). About fifty amino acid residues downstream from the N-termini of all V-PPases are completely different from each other, although the amino acid sequence of the other regions of ORFs are highly conserved (Fig. 2). The diversity among the amino acid sequences in the N-terminal regions may reflect the species and/or organ specificity of each gene. The four contiguous glutamic acid residues (EEEE) present just behind the non-homologous N-terminal region are conserved among all V-PPases (Fig. 2). It is possible that this cluster of the acidic residues has an important part in the enzyme reaction. We predicted the transmembrane-segments on the basis of Klein's method [17]. Consequently, fourteen putative transmembrane segments were found in each polypeptide (Fig. 2). Although the primary structures in the N-terminal regions have low homology, the hydropathy profiles and the transmembrane segments are very similar. It is considered that these peptides have the same conformations.

The $D(X)_7KXE$ motifs which were homologous to the sequence of the catalytic domain of the yeast soluble PPase was found in the sequences of AVP and the

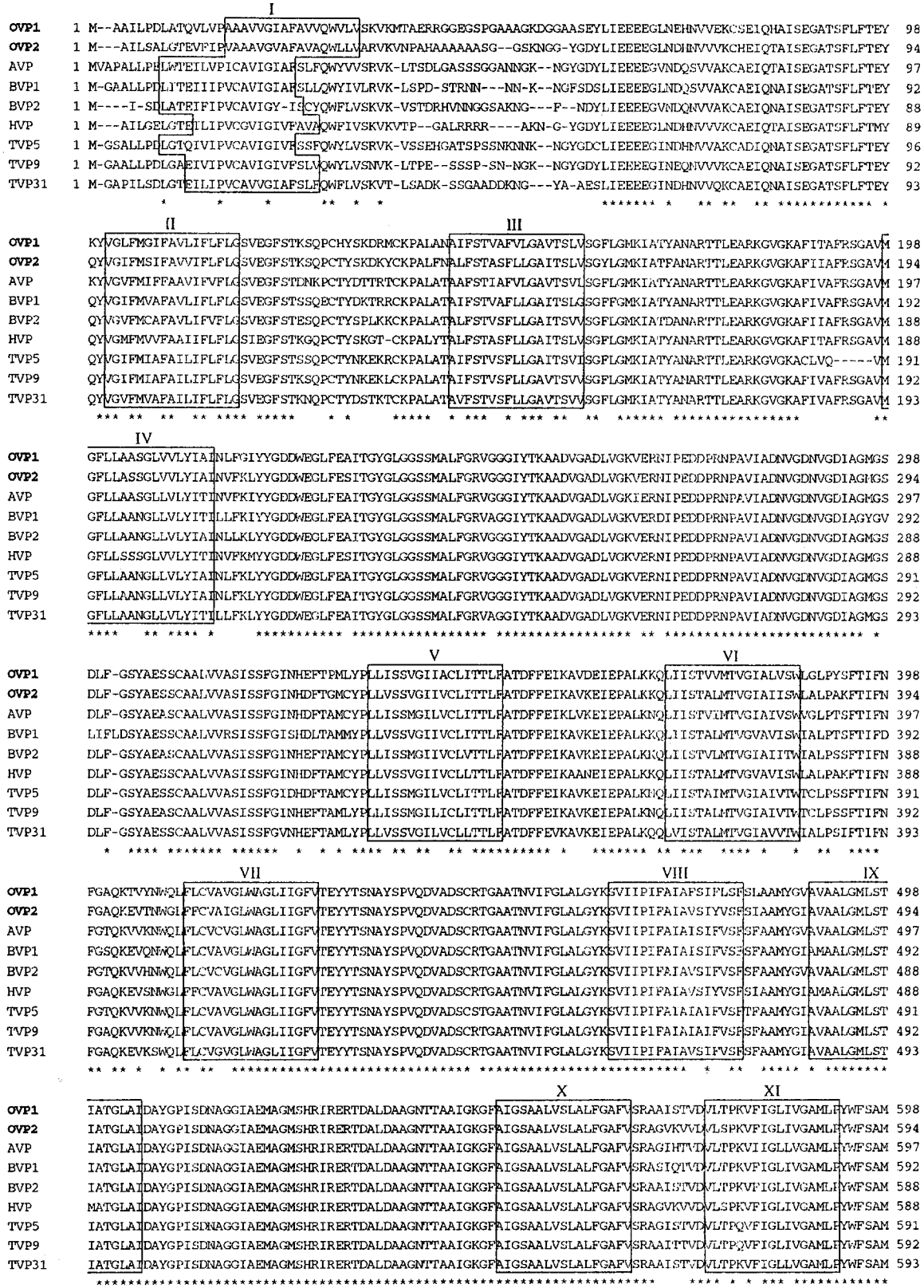

Fig. 2. Comparison of the deduced amino acid sequence of OVP (*O. sativa*) with those of AVP (*A. thaliana*) [37], BVP (*B. vulgaris*) [16], HVP (*H. vulgare*) [43] and TVP (*N. tabacum*) [20]. Dashes show gaps introduced to maximize alignment using multi-alignment from the GENETYX version 7.0. Amino residue numbers beside the sequences exclude gaps. Residues identical in all sequences are indicated by asterisks. Boxed regions show locations of possible transmembrane segments predicted by the PSORT program [25].

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                                                                      XII                    XIII
OVP1   TMKSVGSAALKMVEEVRRQFNSIPGLMEGTTKPDYATCVKISTDASIKEMIPPGALVMLSPLIVGIFFGVETLSGLLAGALVSGVQIAISASNTGGAWDN 698
OVP2   TMKSVGSAALKMVEEVRRQFNSIPGLMEGTGKPDYATCVKISTDASIKEMIPPGALVMLTPLIVGTLFGVETLSGVLAGALVSGVQIAISASNTGGAWDN 694
AVP    TMKSVGSAALKMVEEVRRQFNTIPGLMEGTAKPDYATCVKISTDASIKEMIPPGCLVMLTPLIVGFFFGVETLSGVLAGSLVSGVQIAISASNTGGAWDN 697
BVP1   TMKSVGSAALKMVEEVPKQFNTIPGLLEGTAKPDYATCVKISTDASIKEMIPPGALVMLTPLIVGTFFGVETLSGVLAGSLVSGVQIAISASNTGGAWDN 692
BVP2   TMKSVGSAALKMVEEVRRQFNTIPGLMEGTAKPDYATCVKISTDASIKEMIPPGALVMLTPLIVGIFFGVETLSGVLAGSLVSGVQIAISASNTGGAWDN 688
HVP    TMKSVGSAALKMVEEVRRQFNTIPGLMEGTAKPDYATCVKISTDASIKEMIPPGALVMLTPLIVGTLFGVETLSGVLAGALVSGVQIAISASNTGGAWDN 688
TVP5   TMKSVGSAALKMVEEVRRQFNTIPGLMEGLAKPDYATCVKISTDASIKEMIPPGALVMLTPLIVGIFFGVETLSGVLAGALVSGVQIAISASNTGGAWDN 691
TVP9   TMKSVGSAALKMVEEVRRQFNTIPGLMEGTAKPDYATCVKISTDASIKEMIPPGALVMLTPLIVGIFFGVETLSGVLAGALVSGVQIAISASNTGGAWDN 692
TVP31  TMKSVGSAALKMVEEVRRQFNTIPGLMEGTAKPDYATCVKISTDASIKEMIAPGALVMLTPLIVGILFGVETLSGVLAGSLVSGVQIAISASNTGGAWDN 693
       ***************  *** **** **  ******************* ** **** *****  ******** *** ********************

                                                           XIV
OVP1   AKKYIEAGASEHARTLGPKGSDCHKAAVIGDTIGDPLKDTSGPSLNILIKLMAVESLVFAPFFATHGGILFKWF 771
OVP2   AKKYIEAGASEHARTLGPKGSDCHKAAVIGDTIGDPLKDTSGPSLNILIKLMAVESLVFAPFFATHGGLLFKWF 767
AVP    AKKYIEAGVSEHAKSLGPKGSEPHKAAVIGDTIGDPLKDTSGPSLNILIKLMAVESLVFAPFFATHGGILFKYF 770
BVP1   AKKYIEAGASEHARTLGPKGSDAHKAAVIGDTIGDPLKDTSGPSLNILIKLMAVESLVFAPFFATHGGLLFKYL 765
BVP2   AKKYIEAGASEHARSLGPKGSEPHKAAVIGDTIGDPLKDTSGPSLNILIKLMAVESLVFAPFFATHGGLLFKLF 761
HVP    AKKYIEAGNSEHARSLGPKGSDCHKAAVIGDTIGDPLKDTSGPSLNILIKLMAVESLVFAPFFATYGGLLFKYI 761
TVP5   AKKYIEAGASEHARTLGPKGSEPHKAAVIGDTIGDPLKDTSGPSLNILIKLMAVESLVFAPFFATHGGILFKIF 764
TVP9   AKKYIEAGASEHARTLGPKGSDPHKAAVIGDTIGDPLKDTSGPSLNILIKLMAVESLVFAPFFATHGGLLFKIF 765
TVP31  AKKYIEAGVSEHARTLGPKGSDAHKAAVIGDTVGDPLKDTSGPSLNILIKLMAVESLVFAPFFATHGGLLFKLF 766
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Fig. 2 (continued).

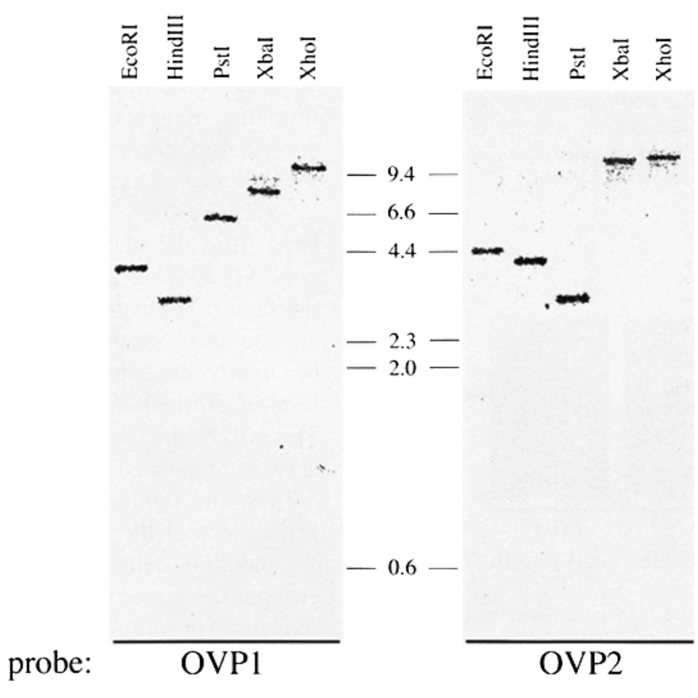

Fig. 3. Genomic Southern analysis of *OVP1* and *OVP2*. 5 μg of genomic DNA of rice (cv. Nipponbare) was digested with *Eco*RI (lane 1), *Hin*dIII (lane 2), *Pst*I (lane 3), *Xba*I (lane 4) or *Xho*I (lane 5). The digested DNA was separated by gel electrophoresis, blotted and hybridized to ^{32}P-labelled 3′-UTR of *OVP1* or *OVP2*. The molecular size marker (kb) is indicated.

other V-PPases [16, 20, 32]. OVP1 and OVP2 have the $D(X)_7KXE$ motif at positions 258 and 254, respectively (Fig. 2). The NEM-reactive residue, Cys-634, [15, 46] also exists in the sequences of OVP1 and OVP2 at positions 636 and 632, respectively. On the other hand, the V-PPase is inhibited by dicyclohexylcarbodiimide (DCCD) [23] and the $EXI(X)_4L(X)_6LF$ motif, of which the glutamate is a DCCD-reactive residue, was proposed [28] by the sequence comparison between the V-PPases and the c-subunit of F_0F_1-

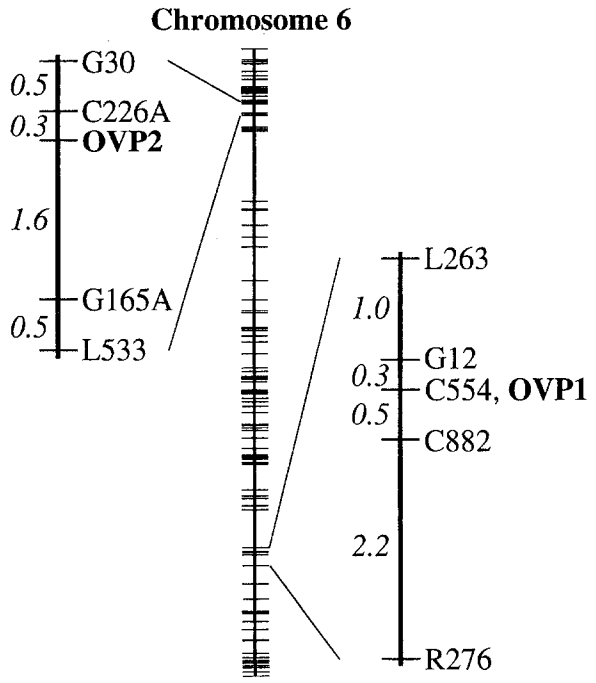

Fig. 4. Chromosomal location of *OVP1* and *OVP2*. A portion of chromosome 6 is represented schematically with the positions of *OVP1* and *OVP2* relative to previously mapped markers [19]. Italic numbers indicate the distance between loci by cM.

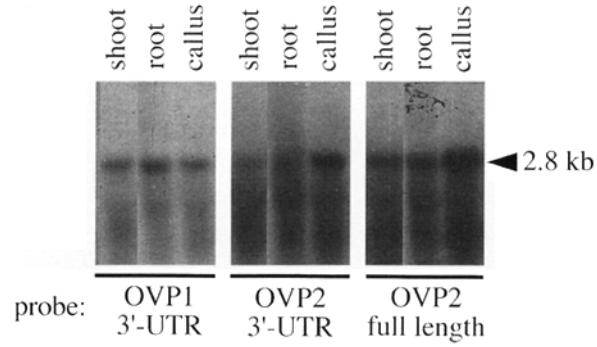

Fig. 5. Northern analysis of rice V-PPases. Each 10 μg of total RNAs isolated from shoots and roots of 10-day-old rice and 7-day-cultured rice calli was electrophoresed, blotted and hybridized to the ^{32}P-labelled probe. a, the 3′-untranslated region (UTR) of *OVP1* was used as an OVP1-specific probe; b, the 3′-UTR region of *OVP2* was used as an OVP2-specific probe; c, the full-length of *OVP2* was used as a non-specific OVP probe. The size of the transcripts is ca. 2.8 kb.

ATPases. Its residue is found at positions 230 and 226 in the sequences of OVP1 and OVP2. Although this motif in the sequence of the c-subunit of F_0F_1-ATPases is localized in a transmembrane segment, the corresponding regions of OVP1 and OVP2 show low hydrophobicity, and the $EXI(X)_4L(X)_6LF$ sequence is located outside the putative transmembrane segments predicted by Klein's method (Fig. 2). To determine the binding site of DCCD, further studies such as site-directed mutagenesis are needed.

We determined the chromosomal locations of *OVP1* and *OVP2* encoding the V-PPase of rice (Fig. 4). A single locus was found for each gene and both were localized in chromosome 6. The V-PPases are thus considered to form a multigene family as well as other primary ion translocases such as the plasma membrane and the vacuolar H^+-ATPases. There are more than 10 isoforms of the plasma membrane H^+-ATPase in *Arabidopsis* [39] and at least seven isoforms in tomato [8]. We further isolated two clones (classes III and IV) partially encoding the V-PPase from the rice cDNA library (data not shown), so that at least four isoforms seem to be present in rice cells. A highly conserved colinearity of genes between rice and wheat [18] and conserved homologies between rice and maize [1] are well established. The existence of a multigene family of the V-PPase in the rice genome and the structural similarity of genomes among these monocots show that other monocots may have several isoforms of the V-PPase as well as rice.

Northern analysis shows that *OVP1* and *OVP2* isolated from the cDNA library constructed from the poly(A)$^+$ RNA of calli are also expressed in the intact rice plants. The expression patterns of *OVP1* and *OVP2* are similar in intact rice cells (Fig. 5). Both seem to be slightly less expressed in shoots than in roots. The level of expression in the calli of *OVP2* is very high. This will be the reason why more clones of the OVP2 class were isolated than those of the OVP1 class. We did not find significant differences between the gene expressions of the two isoforms in the intact plants. We should investigate with mRNA isolated from other organs (e.g., stems, leaves, flowers and developing seeds) and in various developmental stages using more precise techniques (e.g., immunolocalization analysis and β-glucuronidase assay of transgenic plants) to analyze organ- or tissue-specific expression. The different extent of mRNA in calli between the isoforms implies that these genes are regulated in a different manner. Carystinos *et al.* [4] showed that the V-PPase of rice was induced by anoxia. The calli used in this experiment were cultured in liquid media. *OVP2* might be the isoform responsive to anoxia stress.

Our previous investigations [26, 40] revealed several relationships between the pyrophosphate (PPi)-dependent H^+-pumping and physiological phenome-

na: (1) vacuolar alkalization observed in salt-stressed roots of mung bean may be related to the inhibition of H^+-translocation by the V-PPase, whereas cytoplasmic pH is thought to be regulated by stimulation of the plasma membrane H^+-ATPase [26]; (2) the V-PPase probably acts as the major pump during germination and/or at early developmental stages in pumpkin cotyledons, and a decrease in PPi-dependent activities is one of the earliest events in the senescence of the cotyledons [40]; (3) PPi-dependent H^+-translocation of some rice cultivars which are sensitive to chilling is remarkably decreased by cold treatment, although ATP-dependent H^+-translocation across their tonoplasts is less influenced (data not shown). These data show that not only a function of H^+-ATPases or V-PPase but also interactions among both H^+ pumps may play important roles in many physiological processes. We have also isolated two cDNAs encoding the plasma membrane H^+-ATPase from rice [29, 44]. Studies by gene engineering and biochemical methods using these clones should help to define the meaning of the existence of each H^+ pump and explain how to regulate their activities cooperatively in plant cells.

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