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Molecular Cloning, Water Channel Activity and Tissue Specific Expression of Two Isoforms of Radish Vacuolar Aquaporin [1]

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A major membrane intrinsic protein (VM23) in vacuoles of radish (*Raphanus*) tap root was investigated. The cDNAs for two isoforms of VM23, γ- and δ-VM23, encode polypeptides of 253 and 248 amino acids, respectively. γ- and δ-VM23 correspond to the γ- and δ-TIP (tonoplast intrinsic protein) of *Arabidopsis*. The deduced amino acid sequences of the two VM23 isoforms were 60% identical. The amino-terminal sequence of γ-VM23 showed agreement with the direct sequence of the purified VM23, suggesting that γ-VM23 is the most abundant molecule among the VM23 isoforms. When mRNAs of γ- and δ-VM23 were injected into *Xenopus* oocytes, the osmotic water permeability of oocytes increased 6-fold (60 to 200 $\mu m\ s^{-1}$) of the control oocytes. The transcripts of both isoforms were detected in a high level in growing hypocotyls and young leaves, but δ-VM23 was not detected in seedling roots. Light illumination enhanced the transcription of two genes of VM23 in cotyledons and roots but suppressed their expression in hypocotyls the growth of which was inhibited by light. These findings suggest that the expression of VM23 is tightly related to cell elongation.

Key words: Aquaporin — Radish (*Raphanus sativus*) — Vacuolar membrane — Water permeability — *Xenopus* oocyte.

Aquaporin is a novel channel protein that facilitates the water transport across biomembranes in an osmotic pressure-dependent manner (Chrispeels and Agre 1994, Maurel 1997). Aquaporin belongs to a ubiquitous membrane intrinsic protein (MIP) family that is distributed in animals, plants and bacteria (for review; Chrispeels and Agre 1994, Agre et al. 1995, Maurel 1997). The MIP family members have six membrane-spanning domains and two sets of the perfectly conserved motif, Asn-Pro-Ala. Some of the family members have been demonstrated to selectively transport water, glycerol, urea, and small molecules, respectively (Agre et al. 1995, Maurel 1997). In red cells and renal cortex, aquaporins account for a few percentage of the plasma membrane proteins (Nielsen et al. 1993). Recently, the three-dimensional organization (tetramer) of red cell aquaporin has been shown by electron crystallography of the two-dimensional crystals (Walz et al. 1997, Cheng et al. 1997).

Plant aquaporins possess some unique characteristics. (i) Two types of aquaporins are located in the plasma and vacuolar membranes. They are slightly different in molecular size and primary sequence (Park and Saier 1996, Maurel 1997). Nod26, a nodule-specific symbiosome membrane protein, is another type of plant aquaporin, and has been demonstrated to have ion channel activity (Weaver et al. 1994). (ii) Vacuolar aquaporin is significantly abundant in land plants, such as radish, mung bean, *Arabidopsis thaliana* and beet (Johnson et al. 1989, Maeshima 1992, Marty-Mazars et al. 1995). It is similar to the band 3 protein (anion antiporter) in red cells. (iii) There are many isoforms of aquaporin in both plasma and vacuolar membranes (Park and Saier 1996, Maurel 1997). *Arabidopsis* has 23 isoforms of aquaporins (Weig et al. 1997). The physiological meaning of the high content of vacuolar aquaporin, the differences in the physiological function of each isoform, and the physiological relationship between vacuolar and plasma membrane aquaporins have not been determined.

In most plant tissues, a large part of the cell is occupied by an enormous central vacuole. Vacuolar membrane aquaporin was found first in *Arabidopsis* as a γ-type tonoplast integral protein (γ-TIP; Höfte et al. 1992, Maurel et al. 1993). Fleurat-Lessard et al. (1997) found a high content of aquaporin in the central vacuole of motor cells of *Mimosa pudica*, which indicates that vacuolar membrane aquaporin functions in living plant cells. Also, the major role of aquaporins in osmotic water transport has been demonstrated in plants (Tazawa et al. 1996, 1997, Maggio and Joly 1995, Maurel 1997). We also conducted a

Abbreviations: H^+-PPase, H^+-transporting inorganic pyrophosphatase; MIP, major membrane intrinsic protein; PIP, plasma membrane intrinsic protein; TIP, tonoplast intrinsic protein; V-ATPase, vacuolar H^+-ATPase; VM23, vacuolar integral membrane protein of 23 kDa; WGA, wheat germ agglutinin.

The nucleotide sequences reported in this paper have been submitted to the DDBJ, EMBL and GenBank under accession numbers D84669 (γ-VM23, VIP1) and AB010416 (δ-VM23, VIP3).

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series of experiments on vacuolar aquaporin. A major hydrophobic membrane protein in vacuoles of radish tap root is VM23 (Maeshima 1992). The relative level of VM23 in the vacuolar membrane increases during seed germination and tissue growth (Maeshima 1990, Maeshima et al. 1994, 1996). Vacuolar membranes and membrane enzymes can be easily isolated in a high quality from radish tap root and used for the analysis of VM23 at the molecular biological and protein chemical levels. In this study, we sequenced cDNAs for two isoforms of VM23, γ- and δ-VM23, and demonstrated their water channel activity by the *Xenopus* oocyte assay system. Also, we found a difference in the gene expression in radish between γ- and δ-VM23.

Materials and Methods

Plant materials—Radish (*Raphanus sativus* L. cv. Tokinashidaikon) seeds were imbibed with water and then germinated on a net floated on 0.5 mM $CaSO_4$ in the dark at 26°C for 5 d. Primary roots composed of hypocotyl and root were removed from etiolated seedlings and used for RNA preparation. In some experiments, radish plants were grown on the Nagoya University farm or in moist vermiculite in a greenhouse.

Vacuolar membrane preparation and affinity blotting with WGA—Vacuolar membranes and VM23 were purified from radish taproots as described previously (Maeshima 1992). SDS-PAGE was performed in 12% gels by the standard method. To determine the affinity of VM23 to WGA, the purified VM23 (10 μg) was separated by SDS-PAGE and blotted onto a PVDF membrane. The membranes were incubated with peroxidase-linked WGA (5 $\mu g\ ml^{-1}$, Wako) in the presence or absence of 0.5 M *N*-acetylglucosamine. WGA-binding proteins on a PVDF membrane were visualized with ECL Western blotting reagents (Amersham).

RNA extraction, cDNA construction and cDNA screening—Fifty grams of radish tissue was frozen in liquid nitrogen and ground with a motor and pestle. RNA was extracted from frozen powder by the method of phenol/SDS extraction method, and then mRNA was purified by oligo(dT)-cellulose column chromatography. A cDNA library was synthesized from 5 μg of mRNA, ligated into Uni-ZAP XR phage vector (Stratagene), and then packaged with Gigapack II Gold packaging extract (Stratagene). cDNA templates were amplified to obtain a DNA probe by polymerase chain reaction using *pfu* DNA polymerase and gene-specific primers (forward, 5′-CA(A/G)GA(A/G)GA(A/G)-ACIACICA(C/T)CC-3′; reverse, 5′-ACGGTGTAAACAAGCC-CGAATGT-3′). The forward and reverse primers correspond to the N-terminal part of VM23 (QEETTHP; Maeshima, 1992) and the nucleotide sequence of the fourth membrane domain of γ-TIP (TFGLVYTV; Höfte 1992), respectively. Amplification protocol was 3 min at 95°C (once), 1 min at 95°C, 1 min at 50°C, 2 min at 72°C (40 cycles), and 7 min at 72°C (once). The amplified DNA fragment (470 bp) was purified, and then ligated into the *Hinc*II site of pUC119 plasmid vector for transformation of *E. coli* MV1184. The insert DNA was confirmed to be a cDNA fragment of VM23 by DNA sequencing. This DNA probe was radio-labeled with α-^{32}P-dCTP by the random priming method (Megaprime labeling kit, Amersham). Recombinant phages from the library were blotted on a nylon membrane, and then screened with the labeled probe DNA by the standard hybridization method (Sambrook et al. 1989). Positive clones were in vivo excised into the pBluescript SK(−) plasmid (Stratagene). The DNA sequence was determined from single-strand plasmid DNAs by the dideoxy chain termination method using T7 polymerase. Templates were prepared as single strand DNA from pBluescript SK(−) plasmid with helper phage M13K07. Nucleotide sequences were analyzed using a DNA sequencer (Pharmacia). The obtained sequences were analyzed using the DNASIS program of the Hitachi Software Engineering.

RNA analysis—Total RNA fractions were isolated from several organs of 4-d-old seedlings and radishes grown for 7 weeks. One gram of the tissues was frozen in liquid nitrogen, and then mixed with 10 ml of 4.2 M guanidium thiocyanate, 0.5% *N*-sodium lauroyl sarkosyl, 25 mM sodium citrate, 0.1% antifoam A emulsion, and 1% β-mercaptoethanol. After centrifugation at 12,000 rpm for 5 min, RNA was extracted from the supernatant by the phenol/chloroform method. Finally, RNA was dissolved with a small volume of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. Aliquots of the RNA fractions were electrophoresed in a 1.0% agarose gel, and then transferred to a nylon membrane. The membrane was prehybridized in a hybridization buffer (0.5 M Na_2HPO_4, pH 7.2, 1 mM EDTA, 7% SDS, and 0.02% salmon sperm DNA) at 65°C for 1 h, and was then hybridized with ^{32}P-labeled probe DNA (10^6 cpm ml^{-1}) at 65°C for 20 h. DNAs containing the 5′-untranslated sequences of VIP1 (327 b, *Eco*RI-*Hpa*I fragment) and VIP3 (220 b, *Sma*I-*Sma*I fragment), respectively, were used as probes. The membrane was washed twice in 0.1 × SSC containing 0.1% SDS at 65°C for 1 h, exposed to an imaging plate (Fuji Film), and then its radio-image was analyzed with a Fuji image analyzer BAS2000-II.

Genomic southern hybridization—DNA was extracted from 2-d-old seedlings with cetyl trimethyl ammonium bromide. Two μg of DNA was digested by *Bam*HI, *Eco*RI, *Eco*RV or *Hin*dIII, electrophoresed on a 0.8% agarose gel, and then transferred to a nylon membrane. The membrane was prehybridized, and then hybridized with 10^6 cpm ml^{-1} ^{32}P-labeled probe DNA at 65°C for 20 h. The membrane was washed twice in 0.1 × SSC containing 0.1% SDS at 65°C for 30 min, and then exposed to an imaging plate. The DNA fragments of VIP1 and VIP3 without 5′-noncoding region were used as probes.

In vitro RNA synthesis—DNAs that contained the entire coding sequences of γ- and δ-VM23 were formed by ligation of a *Nco*I DNA fragment, and then cloned into the *Nco*I site of pSPUTK with an in vitro translation vector (Stratagene) carrying the 5′ and 3′ untranslated sequences of the β-globin gene of *Xenopus laevis* (Preston et al. 1992). Capped RNAs encoding γ- and δ-VM23 were synthesized in vitro using a MEGAscript transcription kit (Ambion). Transcription was performed at 30°C for 4 h in a reaction medium composed of 5 mM ATP, 5 mM CTP, 5 mM UTP, 1 mM GTP, 4 mM m^7G(5′)ppp(5′)Gp cap analog (Pharmacia), enzyme mixture, transcription buffer, and cDNA. A rabbit reticulocyte lysate system (Amersham) was used for in vitro translation of capped mRNAs in the presence of ^{35}S-methionine (Amersham) to confirm that they were translatable mRNAs.

Assay of osmotic water permeability in a Xenopus oocyte system—Osmotic water permeability of VM23 was assayed in *Xenopus* oocytes as described previously (Sabirov et al. 1998). Barth's saline (BS) was sterilized before use. The Ca-free BS contained 88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, and 15 mM HEPES-NaOH, pH 7.5. Standard BS was prepared by adding 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 10 $\mu g\ ml^{-1}$ sodium penicillin, and 10 $\mu g\ ml^{-1}$ streptomycin sulfate to a Ca-free BS. Stage V and VI oocytes were isolated from an ovary of a mature frog, and then defolliculated by treatment with collagenase

(Sigma, type I; 1 mg ml^{-1} for 3–4 h) in a Ca-free BS. Cells were injected with 75 nl of in vitro transcribed RNAs (250–300 ng ml^{-1}) or distilled water by a "Nanoject" automatic microinjector (Drummond Scientific), and then incubated at 20°C for 2–4 d in the standard BS. Oocytes were viewed by transmitted light on an inverted microscope (Nikon) and imaged on a CCD camera (C2400, Hamamatsu Photonics). Data were recorded on an optic disk recorder (LQ3100A, Panasonic) and simultaneously analyzed online using a frame grabber, VISIONplus-AT (Image Technology Inc.), controlled by an Intel i-486 based computer (AST). Images were collected every 5 s. Volume changes were calculated from changes in cross-sectional area A using relationship $V/V_o=(A/A_o)^{3/2}$, where V_o and A_o are the initial volume (cm^3) and area (cm^2), V and A are their corresponding values at time t. To avoid the difference in the ionic strength and the concentration of electrolytes between isotonic and hypotonic solutions, oocytes were first bathed for at least 3 minutes in an isotonic BS composed of 15 mM NaCl, 146 mM mannitol, 15 mM HEPES-NaOH, 1 mM KCl, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 0.3 mM $Ca(NO_3)_2$, and 0.41 mM $CaCl_2$, and then moved to hypotonic BS, which was made by removing 146 mM mannitol from isotonic BS. Osmolarities of isotonic BS and hypotonic BS measured by an osmometer (OM-801, Vogel) were 207 and 61 mosM, respectively. Osmotic water permeability was obtained from the following equation:

$$P_f=[V_o/(S\times V_w\times \Delta\Pi)]\,[d(V/V_o)/dt]$$

where V_o is the initial oocyte volume, S the oocyte surface area (0.045 cm^2), V_w the partial molar volume of water (18 $cm^3\,mol^{-1}$), $\Delta\Pi$ the osmotic gradient (146 mosM in our experimental conditions), and $d(V/V_o)/dt$ the rate of relative cell volume change. The oocyte volume under the recording system was calibrated with certified particle-size standards (glass microspheres, Duke Scientific Co.). Experiments were performed at room temperature (23–25°C). Data are presented as the mean values ± SE. The number of experiments was more than 9 for each set of transcripts.

Results

Molecular cloning of VM23 cDNA—To analyze the physiological function of the major membrane protein VM23, we constructed a cDNA library for mRNAs in radish seedlings. Among 10,000 recombinant phages, DNAs of 28 phages were hybridized with a DNA probe of VM23. Three DNAs of the clones encoded different polypeptides. We denoted them VIP1, VIP2 and VIP3. The nucleotide sequence of VIP2 was homologous to that of VIP1 but not VIP3. The cDNA of VIP1 consists of 1,054 bp upstream of the polyadenylate tail, which includes a 70-bp 5′ leader sequence, followed by 759 bp of an open reading frame encoding 253 amino acids, and a 225-bp 3′-noncoding region. The VIP3 cDNA is comprised of 911 bp upstream of the polyadenylate tail, which includes a 41-bp

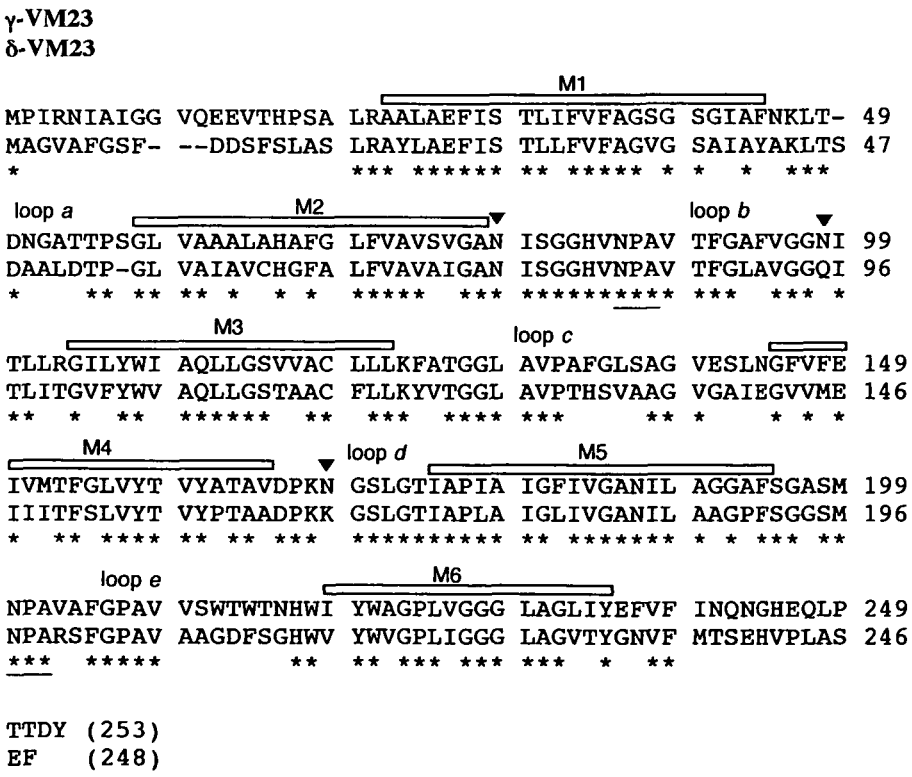

Fig. 1 Comparison of the primary sequences between γ- and δ-VM23. Stars under the sequences indicate the identical residues. Two sequences were 60% identical. Open bars mark six putative membrane spanning domains (M1 to M6). Parts between the membrane-spanning domains are shown as loops *a* to *e*. NPA motifs are underlined. Closed triangles show putative *N*-glycosylation Asn residues. The accession numbers of γ- and δ-VM23 are D84669 and AB010416, respectively.

5′ leader sequence, followed by 744 bp of an open reading frame encoding 248 amino acids, and a 126-bp 3′-noncoding region. Adenylic acids in the polyadenylate tails of VIP1 and VIP3 were 31 and 64 residues, respectively.

Structural characteristics of the deduced VM23 polypeptides—The calculated molecular weights of the polypeptides derived from VIP1 and VIP3 were 25,831 and 24,911, respectively, which were slightly larger than the value for the purified VM23 on SDS-PAGE. The deduced amino acid sequence of VIP1 was 60% identical to that of VIP3 (Fig. 1). At the amino acid level, VIP1 and VIP3 were 90 and 95% identical to γ- and δ-TIP of *Arabidopsis*, respectively. The nucleotide sequences of VIP1 and *Arabidopsis* γ-TIP gave 83.5% homology within their coding regions. VIP3 and cotton δ-TIP (Ferguson et al. 1997) gave 83% homology. Thus, the polypeptides encoded by VIP1 and VIP3 were registered as γ- and δ-VM23, respectively.

The sequence of the N-terminal part of γ-VM23, but not δ-VM23, was in agreement with the direct sequence of the purified VM23 (Maeshima 1992). The purified preparation was the mixture of some species of VM23 including γ- and δ-VM23. When the purified VM23 (585 pmol) was subject to N-terminal amino acid sequence analysis, the main residue (60–100% in yield) at each cycle was consistent with the residue of γ-VM23. Namely, the γ-VM23 accounts for more than 60% of VM23 in the vacuolar membrane of radish tap root. This suggests that γ-VM23 is an abundant species of VM23 in radish vacuolar membranes. VM23 is the major protein of vacuolar membrane as shown in Figure 2A. VM23 was stained strongly by silver rather than Coomassie blue. Judging from the densitometrical analysis of the intensity of VM23 bands (monomer and dimer) in this silver-stained gel, VM23 was estimated to account for about 40% of the vacuolar membrane of radish tap root. Thus, the amount of γ-VM23 may be more than a quarter of the vacuolar membrane protein.

The deduced sequences were analyzed by the method of Kyte and Doolittle (1982). Both VM23 sequences had six hydrophobic domains of sufficient length to span a membrane (Fig. 1). γ- and δ-VM23 had two sets of a characteristic sequence NPA in loops *b* and *e*. The first NPA motif was found in a sequence of SGGHXNPAVT which is a signature sequence of the MIP family. Short parts comprising the NPA motif in loops *b* and *e* are relatively hydrophobic, thus they may be buried in the membrane but not span it. By site-directed mutagenesis of *Arabidopsis* γ- and δ-TIP, Daniels et al. (1996) identified the mercury-sensitive site as Cys-116 and Cys-118 for γ- and δ-TIP, respectively. Cysteine residues in the third membrane-spanning domain of γ- (Cys-119) and δ-VM23 (Cys-116) were conserved in VM23.

A motif Asn-X-Ser/Thr is known to be a potential *N*-glycosylation site. The polypeptides have three or single potential glycosylation residues of asparagine at positions

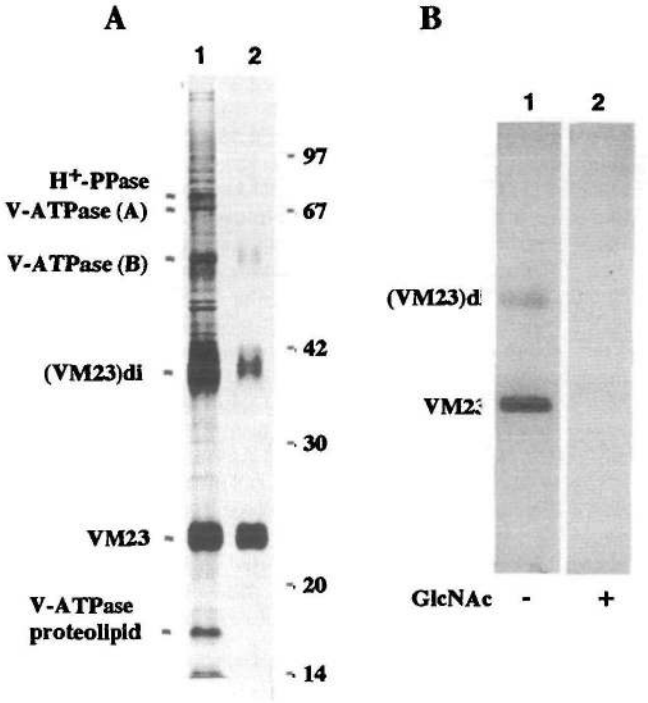

Fig. 2 Abundance of VM23 in radish vacuolar membranes (A) and affinity blotting of VM23 with WGA (B). (A) Vacuolar membranes (1 μg, lane 1) and the purified VM23 (0.25 μg, lane 2) were subjected to SDS-PAGE and then the gel was silver stained. Molecular masses of the standards are given in kDa on the right. The positions of H^+-PPase (73 kDa), three subunits of subunits of V-ATPase (68, 57 and 16 kDa), VM23 (23 kDa), and its dimeric form [(VM23)di, 40 kDa] are indicated on the left. (B) The purified VM23 (10 μg) was subjected to SDS-PAGE and blotted onto a PVDF membrane. The membrane slits were incubated with peroxidase-linked WGA in the presence (lane 2) or absence (lane 1) of 0.5 M *N*-acetylglucosamine.

79, 98 and 169 in γ-VM23 and at position 77 in δ-VM23. Indeed, WGA reacted with both monomeric and dimeric forms of VM23 (Fig. 2B). *N*-acetylglucosamine competitively inhibited the binding of this lectin to VM23 at 0.5 M, suggesting the specific reaction of WGA to VM23.

Water channel activities of γ- and δ-VM23—To determine whether VM23 functions as a water channel, *Xenopus* oocytes were injected with the in vitro transcribed mRNAs for γ- and δ-VM23. The 5′ cap structure was introduced to mRNAs to enhance both the stability and translational efficiency of the microinjected mRNA. It was confirmed using a cell-free translation system that the capped mRNA was translated to a protein of 23 kDa (Fig. 3A). Oocytes were injected with the capped mRNAs and were then incubated for 3 d. We determined the osmotic water permeabilities of oocytes after transfer from a normal solution of 207 mosM to a hypotonic buffer of 61 mosM. Water permeabilities were higher than 100 μm s^{-1} in sever-

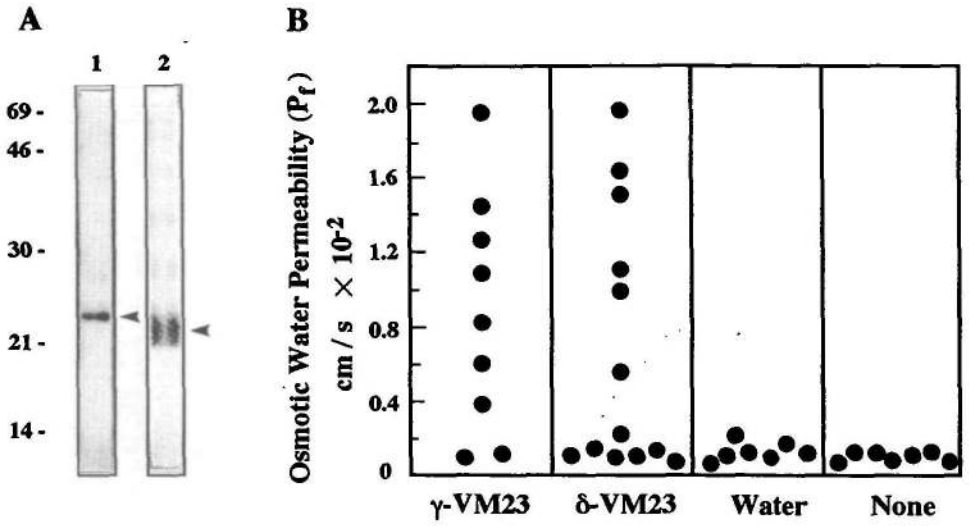

Fig. 3 Water permeability of *Xenopus* oocytes injected with mRNAs for γ- and δ-VM23. (A) The capped mRNAs for γ- (lane 1) and δ-VM23 (lane 2) were translated in vitro with a rabbit reticulocyte lysate and ^{35}S-methionine, and then the translation products were subjected to SDS-PAGE. The arrowheads show the positions of γ- and δ-VM23, respectively. (B) The capped mRNAs for γ- and δ-VM23 were injected to *Xenopus* oocytes. After incubation for 3 d, the injected oocytes (nine eggs for γ-VM23, thirteen eggs for δ-VM23) were assayed for water permeability. The values of water permeability of oocytes injected with the γ- and δ-VM23 mRNAs were 84±20 and 67±19 μm s^{-1}, respectively. Values for the control oocytes injected with water or uninjected were 15±2 and 12±1 μm s^{-1}, respectively.

al oocytes, injected with mRNAs of VM23 (Fig. 3B). The mean values of all oocytes determined for γ- and δ-VM23 were 84 and 67 μm s^{-1}, respectively. Mean values for oocytes injected with water and non-injected oocytes were 15 and 12 μm s^{-1}, respectively.

Expression of γ- and δ-VM23 in different tissues— Levels of VM23 mRNAs were determined on total RNA isolated from a number of distinct tissues in radish plants with a DNA probe specific for individual VM23. In etiolated seedlings, a high level of γ-VM23 mRNA of about 1.2 kb was detected in roots and hypocotyls, but not in cotyledons (Fig. 4). The relative level of δ-VM23 mRNA was high in hypocotyls, but the transcript was not detected in roots or cotyledons. By light illumination, the cotyledons of seedlings became large and green, and the roots grew more rapidly than those of etiolated seedlings. In contrast, the elongation of hypocotyls was suppressed by light. The level of mRNAs for both VM23 in hypocotyls of seedlings grown under constant light, was about 70% of that of etiolated seedlings. In cotyledons and roots, however, light illumination increased the levels of γ-VM23 mRNA. The level of δ-VM23 transcript also increased under light illumination in three experiments, although the gene did not seem to be expressed in roots of seedlings under any conditions used.

Figure 5 shows the levels of VM23 mRNA in radish plants grown for 7 weeks. Both transcripts for γ- and δ-VM23 were detected in immature tap roots, petioles and young leaves (Fig. 5A). In both VM23, however, the tran-

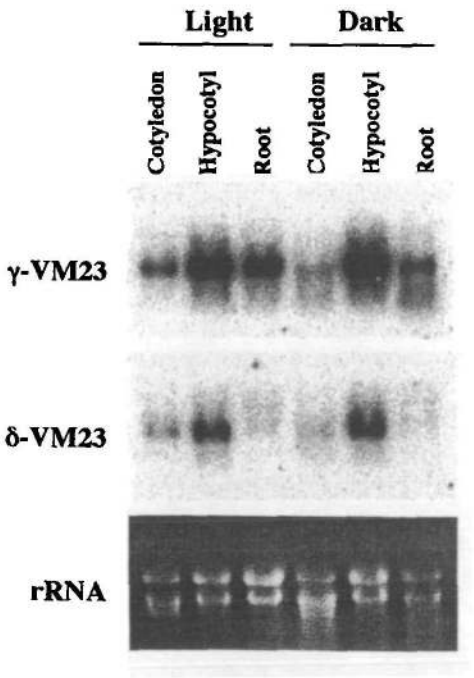

Fig. 4 Levels of transcripts for γ- and δ-VM23 in radish seedlings. Radish seeds were imbibed and germinated in the dark for 4.5 d (right). Other seeds were imbibed in the dark for 1 d, and then germinated under constant light for 3.5 d (left). Total RNA fractions were prepared from 1.5 g of roots, hypocotyls and cotyledons, and then aliquots (10 μg) of them were applied to northern analysis with DNA probes for γ- and δ-VM23. The gel was stained with ethidium bromide to detect rRNAs.

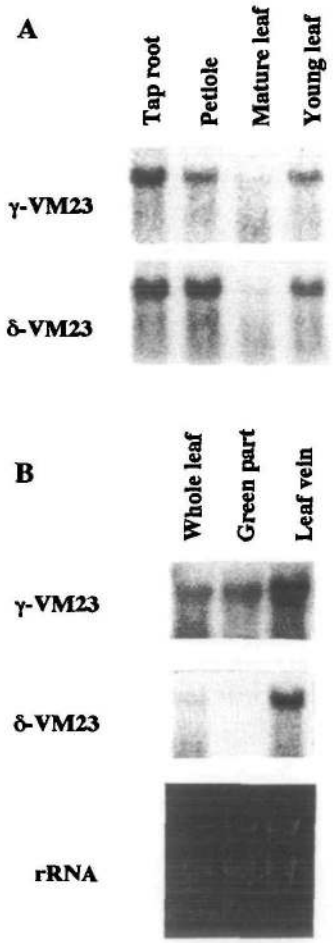

Fig. 5 Levels of mRNA of γ- and δ-VM23 in 6-week-old radish. Radish was grown in a greenhouse for 6 weeks, at which time the plant had three pairs of true leaves. (A) Aliquots (7.5 μg) of the total RNA fractions from growing tap roots (2 cm in diameter), petioles, the first and second leaf pairs (mature leaf), and the first leaf pair (young leaf), were subjected to northern analysis. (B) The mature leaves were divided into the main leaf vein and green part for preparation of RNA. Aliquots of RNA (6 μg) were used for northern analysis.

scripts were scarcely detected in mature leaves. In order to compare the transcript levels between vascular and parenchyma tissues, we separated mature leaves into the main vein (midrib) and green part. The green part consists of mesophyll, epidermis and minor veins. The amounts of RNA recovered from the main vein and green part on the basis of gram fresh weight were 20 and 60 μg, respectively. mRNA for γ-VM23 was clearly detected in both the leaf vein and green part, while, δ-VM23 mRNA was detected only in the leaf vein (Fig. 5B).

Genomic organization of VM23 genes—Figure 6 shows the radish genomic DNA-blot analysis using individual probes consisting of the 5′ untranslated region of γ- and δ-VM23. The sizes of the hybridizing DNA frag-

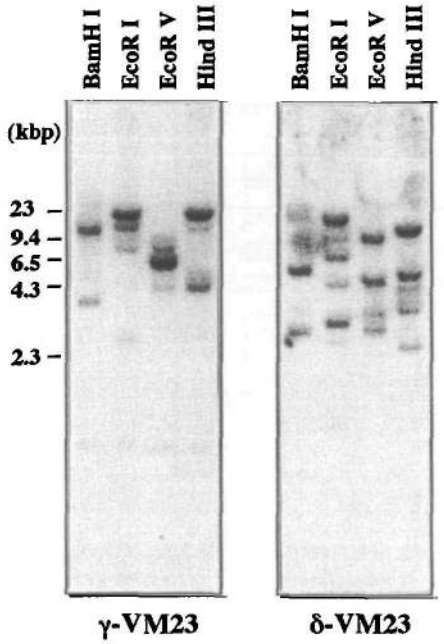

Fig. 6 Genomic southern hybridization analysis of VM23 gene family. Genomic DNA (2 μg) from radish seedlings was restricted with *Bam*HI, *Eco*RI, *Eco*RV, and *Hin*dIII, resolved on a 0.8% agarose gel, blotted to a nylon membrane, and hybridized with probes made from VIP1 (γ-VM23, left panel) and VIP3 (δ-VM23, right panel). Molecular sizes (kb) of standards are shown on the left.

ments were different in γ- and δ-VM23, except for an *EcoR*I fragment of 20 kb. This indicates that there are at least two copies of the γ- and δ-VM23 genes.

Discussion

We cloned the cDNAs of two isoforms of VM23 and determined their water channel activity. γ- and δ-VM23 cloned in this study contain a typical property of an aquaporin family; namely, six putative membrane-spanning domains and an NPA motif in each half of polypeptide. The amino acid sequences of γ- and δ-VM23 had a relatively low homology of 60%, although the two isoforms shared some properties and exhibited the same level of water channel activity. We have recently sequenced two cDNAs for the plasma membrane-type aquaporin of radish. The amino acid sequences of the aquaporins of vacuolar and plasma membranes are less than 40% identical (Suga, Higuchi and Maeshima; unpublished data). We must note that γ- and δ-VM23 were 90 and 95% identical to *Arabidopsis* γ- and δ-TIP, respectively. This suggests that γ- and δ-VM23 (TIP) were separated from an ancestor in the early stage of the molecular evolution, and later the genus of radish was then separated from the genus of *Arabidopsis*.

Animal aquaporins, such as aquaporin-2 are *N*-glyco-

sylated at asparagine-124 in the extracellular hydrophilic loop (Bai et al. 1996). The present study showed the glycosylation of VM23, although the actual glycosylation site or the length of sugar chains have not yet been determined. There are a few potential glycosylation sites in both isoforms as a motif Asn-X-Ser/Thr (Fig. 1). The estimated sites of asparagine residues in the hydrophilic loops *b* and *d* may be exposed to the cytosol judging from its membrane topology. This is inconsistent with the general conception that *N*-linked oligosaccharides are attached to proteins in the ER. Therefore, the glycosylation sites of VM23 must be exposed to the lumenal side of the vacuole. There is a possibility that the third isoform of VM23 has a glycosylated asparagine in the lumenal side of vacuoles. To resolve this contradiction, the actual glycosylation site of VM23 must be determined.

α- and δ-TIP and plasma membrane aquaporins contain common phosphorylation sites, and the phosphorylation is related to regulation of water channel activity (Johansson et al. 1996, Johnson and Chrispeels 1992, Maurel et al. 1995, 1997, Maurel 1997). However, neither γ- nor δ-VM23 has such a phosphorylation site in its structure. Probably, γ- and δ-VM23 may not contain the gate system by which the water channel is switched on or off.

Both isoforms of VM23 from radish exhibited water channel activity in *Xenopus* oocytes. The water channel activity of only three samples of vacuolar aquaporins was determined; namely α-TIP of bean cotyledon (Maurel et al. 1995), *Arabidopsis* γ- (Maurel et al. 1993) and *Arabidopsis* δ-TIP (Daniels et al. 1996). The antibodies specific to VM23 have been used for cell biological studies in many plants, such as tobacco (Matsuoka et al. 1997), pumpkin (Maeshima et al. 1994), pear (Shiratake et al. 1997), and *Mimosa* (Fleurat-Lessard 1997). The findings indicate that the antigens reacting with anti-VM23 antibodies in these plants are actual vacuolar aquaporins. The obtained values for osmotic water permeabilities of γ- and δ-VM23 were among 100 to 200 μm s^{-1}; comparable with those of mammalian and plant aquaporins previously reported (Preston et al. 1992, Fushimi et al. 1993, Daniels et al. 1996). There may be no significant difference in the conductance between VM23 and other aquaporins in plant and animal cells. VM23 accounts for about 40% of radish vacuolar membranes (Fig. 2A). A question arose from the present study why plant cells contain a large amount of aquaporin in the vacuolar membranes. Probably, the vacuoles in growing and mature plants must transport a large quantity of water across the membranes using a small difference of osmotic pressure.

The present study revealed the specificity of VM23 genes. First, both isoforms were expressed in growing tissues, such as roots and young leaves. Second, light illumination enhanced the gene expression of VM23 in roots and cotyledons. Inversely, the expression of VM23 genes was suppressed in hypocotyls under constant light conditions. Third, there is tissue specificity of γ- and δ-VM23 genes, although the tissues expressing the two VM23 isoforms partly overlapped. δ-VM23 was not expressed in young roots or leaf mesophyll, whereas the γ-VM23 transcript was detected in most tissues. This observation is consistent with the high expression of the *Arabidopsis* δ-TIP gene in young shoot, rosette, and flower tissues, but not in root tissue (Daniels et al. 1996).

Here we discuss the relationship of expression of aquaporin genes to cell growth. Northern analysis showed that transcripts of both VM23 were highest in hypocotyls of seedlings. Ludevid et al. (1992) also observed that γ-TIP gene expression was high in the elongating region of *Arabidopsis* roots. In mung bean hypocotyls, the amount of VM23 on the basis of vacuolar membrane protein was low in young cells and increased during cell elongation (Maeshima 1990). Cells of elongating hypocotyls and roots markedly expand in volume within a short time. Rapid incorporation of water into the growing vacuole is necessary for rapid cell expansion, so that the aquaporins facilitate water transport across the vacuolar membranes. VM23 is the most abundant protein in vacuolar membranes. Active transcription of VM23 genes and active synthesis of VM23 are essential for vacuole development in growing cells.

VM23 gene expression was high not only in hypocotyls but also in developing tap roots and young growing leaves (Fig. 5). Light illumination stimulates root growth and cotyledon development. In these tissues, the expression of VM23 genes was enhanced by light. Under a light condition, hypocotyl elongation was inhibited, and VM23 gene expression was suppressed. These and other findings indicate that the gene expression of VM23 and TIP is closely related to the cell elongation. The VM23 gene expression was high in growing roots, but low in leaves. There must be differences in requirement of water transport activity across the vacuolar membranes between roots and leaves. Also, the difference in the expression of VM23 genes between the two organs may be due to the difference in the surface area and the volume of vacuole between roots and leaves.

Aquaporin forms a passive, osmotic pressure-dependent channel for water. Accumulation of inorganic ions, sugars and organic acids increases the vacuolar osmotic pressure, and then a large amount of water is incorporated into vacuoles. The secondary active transport systems for ions and metabolites are activated by a proton motive force generated by vacuolar H^+-ATPase and H^+-PPase. The gene expression of the two proton pumps was highest in the elongating region of hypocotyl of etiolated mung bean seedlings (Nakanishi and Maeshima 1998). We estimate that active syntheses of aquaporins, proton pumps, and secondary active transporters are necessary for development of vacuoles and cells.

In conclusion, VM23 isoforms were expressed in growing tissues, such as roots and young leaves. δ-VM23 was not expressed in young roots and leaf mesophyll, although the γ-VM23 transcript could be detected in most tissues of radish. These findings indicate that the two VM23 genes are independently regulated. Judging from the high expression of VM23 in roots and leaf petioles, the vascular bundle is one of the most important tissues that actively synthesize VM23 proteins. Cells around the xylem and phloem must pass a large quantity of water to parenchyma cells. Whether or not the high content of aquaporin results in the high rate of water transport across the vacuolar membrane is still an unresolved question. In order to deduce the physiological function of each aquaporin isoform, the cell specificity of VM23 gene expression requires further study.

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