Molecular Cloning of a cDNA Encoding Tonoplast Water Channel of Pear Fruit and Its Expression during Development

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Summary

Cloning a cDNA encoding tonoplast water channel of pear fruit disclosed that its deduced amino acid sequence has 77%, 52%, and 50% identities to γ tonoplast intrinsic protein (TIP), δ TIP, and α TIP of Arabidopsis, respectively. Therefore, this clone is considered to be a member of γ TIPs and is designated as *Py-γ TIP*. Deduced amino acid sequence of *Py-γ TIP* has six membrane spanning domains and two NPA motifs, which are conserved within all water channels. Antibody against VM23, which is the vacuolar water channel of radish, recognized two proteins derived from tonoplast proteins of pear fruit yielded a major spot at pI 4.8 and a minor one at pI 5.2 by two dimensional–polyacrylamide gel electrophoresis (2D–PAGE). Putative pl of deduced amino acid sequence of *Py-γ TIP* is 4.86; therefore, the major spot in 2D–PAGE is considered to be a translated product from *Py-γ TIP* which might play a dominant role in fruit. Northern blot analysis showed that *Py-γ TIP* was expressed especially high at 23 and 41 days after flowering (DAF), which correspond to the middle and end of the cell–division stage of fruit development. This suggests that the role of *Py-γ TIP* might be to sustain vacuole biogenesis and to prepare the cells for the rapid increase in vacuolar volume at the cell–division stage of a fruit.

Key Words: fruit development, γ TIP, molecular cloning, pear, tonoplast water channel.

Introduction

Water can pass more easily through the membrane of plant cells than can solutes. Until recently, it was considered that water could move by diffusion across the membrane without the aid of proteins. Wayne and Tazawa (1990) found the evidence involving proteins in water permeability of plant membrane, and Maurel et al. (1993) first identified the water channel in plant, which they called γ tonoplast intrinsic protein (TIP). In subsequent experiments, plasma membrane water channels, called plasma membrane intrinsic proteins (PIPs), were also found in plant cells. To date, many isoforms of water channel were found in plant; their physiological roles had been revealed by degrees (Kjellbom et al., 1999). In general, they are highly expressed in tissues that experiences high water flux, such as vascular tissues, guard cells of stomata, and zone of cell growth (Schaffner, 1998). A transgenic plant with an antisense construct, which targeted the gene encoding PIP, had a root system 5 times larger than that of the control plant (Kaldenhoff et al., 1998). These observations show the importance of water channels in the water flow and uptake by plants.

Water is the most abundant constituent of fleshy fruit, so that, water channels play important roles in their development. A fruit accumulates a large amount of water for its enlargement; most of it is loaded into the vacuoles of fruit cells, depending on their osmotic pressure. When water moves from a vessel to a vacuole, it must pass through two gates, that is, water channels of the plasma membrane and tonoplast. We have already determined the protein level of the tonoplast water channel (VM23P) during the development of a pear fruit (Shiratake et al., 1997a). Tonoplast water channels are generally divided into three groups, α TIP, δ TIP, and γ TIP. α TIP is seed–specific, whereas, δ TIP and γ TIP exist abundantly in vegetative tissues. In our previous study, we could not identify which type of TIP is predominately expressed or if it was fruit specific, since there was little information about VM23P at the molecular level. In this study, we isolated a cDNA encoding tonoplast water channel of pear (Py-γ TIP) and determined its level during fruit development. Hence, the relationship between Py-γ TIP and VM23P is discussed.

Received; July 24, 2000. Accepted; October 13, 2000.

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This work was supported in part by a Grant–in–Aids (nos. 10219203, 09306002 and 11760020) from the Ministry of Education, Science, Sports and Culture of Japan.

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Materials and Methods

Plant materials

Pear (Pyrus communis L. var. sativa DC. ‘La France’) fruits were harvested at different growth stage in 1999 at Anjo, Aichi Prefecture. The flesh was frozen in liquid nitrogen and stored at -80 °C until using for RNA extraction. To isolate tonoplast, fruits at premature stage were harvested and used immediately without freezing.

Isolation of tonoplast vesicles

Crude membranes, which were isolated as described previously (Shirakata et al., 1997b), were subjected to a sucrose density gradient centrifugation and fractionated according to Shirakata et al. (1998). Fractions that had the highest activity of PPase (a marker for tonoplast) were used as a tonoplast preparation for two-dimensional polyacrylamide gel electrophoresis (2D-PAGE).

2D-PAGE and Immunoblotting

2D-PAGE was performed by a modified method of O’Farrell (1975). Isoelectric focusing (IEF) gel consisted of 2.27 g of urea, 0.81 ml of deionized water, 0.55 ml of 10% (w/v) Triton-X 100 and 0.21 ml of 40% Ampholine (pH 9.5–3.5, Amersham Pharmacia Biotech); it was charged in glass capillaries. The gel was exposed to 100 V for 15 min, to 150 V for 30 min, and to 200 V for 30 min, prior to electrophoresis. The sample was heated in 1% (w/v) sodium dodecylsulphate (SDS) at 70 °C for 7 min. A 5–μl of a solution containing 8 M urea, 10% (w/v) Ampholine (pH 9.5–3.5), 12.5% (w/v) Triton-X 100, and 50 mM dithiothreitol (DTT) was added to a 20–μl aliquot of the sample and the mixture applied to IEF gel. Electrophoresis was carried out at 100 V for 16 hr and then at 400 V for 1 hr. After electrophoresis, the gel was incubated in 125 mM Tris–HCl, pH 6.8, 2.3% (w/v) SDS, 10 μg·ml^{-1} bromophenol blue, 10% (w/v) glycerol, and 2 mM DTT at room temperature for 1 hr, then subjected to SDS-PAGE, according to Laemmli (1970).

For immunoblotting, proteins separated by 2D-PAGE were transferred to a cellulose nitrate membrane by a modified method of Towbin et al. (1979). Binding of the antibody against radish VM23 (Maeshima, 1992) was detected colorimetrically with alkaline phosphatase-conjugated antibody that was raised in goat against rabbit immunoglobulin G.

Molecular cloning of Py-γ TIP

The cDNA fragment of TIP was amplified by a nested PCR method using three kinds of primer. One primer is specific for TIPs (primer-1; forward, 5’-TGATCTT(T/C)GT(G/C)TTCGCCGG-3’); the two others are specific for TIPs and PIPs (primer-2; forward, 5’-ATCTC(T/C)GGTGAC(A/G)TIAACCC-3’, primer-3; reverse 5’-G(T/G)CC(G/A/C)(G/A)CCCC(G/A)(T/A)AA(C/T)CCAGTGGP-3’). Primer-1, primer-2, and primer-3 corresponded to the nucleotide sequences coding for first membrane domain, second loop domain, and the end of the sixth membrane domain of TIPs, respectively (Fig. 1). The first PCR was performed, using primer-1, primer-3, and Taq polymerase. The cDNA library, constructed from pear fruit at 100 days after flowering (DAF) by Suzuki et al. (1999), was used as a template. The amplification protocol was 5 min at 95 °C (once), 1 min at 95 °C, 1 min at 56 °C, 2 min at 72 °C (40 cycles), and 7 min at 72 °C (once). The PCR product was used as a template for the second PCR with primer-2 and primer-3. The amplification protocol was the same as above except for the 1 min at 57 °C rather than 56 °C. An amplified DNA fragment (ca. 420 bp) from the second PCR was cloned in pT7BlueT-vector (Novagen). The inserted DNA was confirmed to be a cDNA fragment of TIP by DNA sequencing. The DNA probes were digoxigenin (DIG)-labeled by using a commercial kit (Roche). About 22,000 plaques of the cDNA library were screened by using the DIG-labeled probe. Three positive clones were obtained and confirmed to have the same sequence as Py-γ TIP; the longest one was fully sequenced. DNA sequencing was performed with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer) by using an automatic DNA sequencer (Model 373, Perkin-Elmer).

Extraction of RNA and northern blot analysis

Total RNA was extracted from the flesh of pear fruit for the northern blot analysis by the method of Suzuki et al. (2000), which is a modified phenol–SDS method of Nakajima et al. (1988) combined with the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980).

Formaldehyde agarose gel electrophoresis of total RNA was performed according to Sambrook et al. (1989) and the RNAs were blotted onto nylon membranes. The full length of Py-γ TIP DNA was DIG-labeled for a probe. The membranes were prehybridized by using a blocking reagent (Roche) at 68 °C for 30 min and hybridized with the probe at 68 °C overnight. The membranes were washed twice in 2 × SSC (0.3 M NaCl and 30 mM sodium citrate), containing 0.1% (w/v) SDS at room temperature for 5 min and in 0.1 × SSC, containing 0.1% (w/v) SDS at 68 °C for 15 min. Detection was done by following the supplier’s protocol and using a chemiluminescent substrate, CDP-Star (Amersham Pharmacia Biotech.). The membrane was exposed to X-ray film.

Results and Discussion

Molecular cloning of Py-γ TIP and its characteristics

To isolate a cDNA encoding tonoplast water channel of pear fruit, primers -1, -2, and -3 were designed. A
cDNA fragment of ca. 420 bp was amplified by the nested PCR method, using these primers and the cDNA library of pear fruit as a template. The DNA fragment was cloned and confirmed to be a cDNA fragment of γ-type TIP by DNA sequencing. When the cDNA library of pear fruit was screened with the probe for this DNA fragment, three positive clones were obtained. These three clones were confirmed to have the same sequence; the longest one was fully sequenced (Fig. 1). This clone contains 69 nucleotides of 5'-untranslated region, 759 nucleotides of coding region, 272 nucleotides of 3'-untranslated region, and 20 nucleotides of poly (A) tail. The coding region encodes a putative polypeptide of 252 amino acids whose molecular mass and isoelectric point (pI) were 26,034 and 4.86, respectively. The deduced amino acid sequence has 77%, 52%, 50% and 77% identities to γ TIP (Höfte et al., 1992), δ TIP (Daniels et al., 1996), α TIP (Höfte et al., 1992) of Arabidopsis, and radish γ - VM23 (γ - type TIP of radish, Higuchi et al., 1998), respectively. A phylogenetic tree, based on the deduced amino sequences of this clone and other TIPs, was constructed with the UPGMA program (DNASIS). The tree shows that this clone is a member of γ - type TIPs (Fig. 2). It was designated as “Py - γ TIP” and deposited in DDBJ under the accession number AB048248.

![Image](image_url)

**Fig. 1.** Nucleotide sequence and deduced amino acid sequence of Py - γ TIP. Six putative membrane-spanning domains are underlined (M1 - M6). NPA motif, which is a conserved sequence within all water channel, was shown as [NPA] C in the black box and N with * show a putative cysteine residue of Hg-binding and putative asparagine residues of N-glycosilation, respectively. The termination codon is marked with an asterisk. The primers for nested PCR were shown with arrows (primer-1, -2, and -3).
Fig. 2. Phylogenetic tree based on the deduced amino sequences of Py-γ TIP with other tonoplast water channel. At-γ TIP, At-δ TIP, At-α TIP and γ-VM23 show γ-TIP (accession number, X63552), δ TIP (U39485), α TIP (X63551) of Arabidopsis, and radish γ-VM23 (D84669), respectively.

All members of the water channel are characterized by the presence of six membrane spanning domains and the signature sequence Asn-Pro-Ala, called NPA motif. This motif is present twice in all the proteins. The deduced amino acid sequence of Py-γ TIP has six membrane spanning domains (M1-M6) and two NPA motifs (Fig. 1). The first NPA motif is located in a loop between M2 and M3; the second one is located between M5 and M6. The water transport activity of some water channels, such as γ- and δ-TIPs, is inhibited by mercury. Daniels et al. (1996) identified the mercury sensitive site as Cys-116 of Arabidopsis γ TIP. Cysteine residue in M3 was conserved in the deduced amino acid sequence of Py-γ TIP (Cys-118). Higuchi et al. (1998) reported that γ VM23 is a glycoprotein and Asn-79, -98, and -169 are potential N-glycosilation sites of γ VM23. In the deduced amino acid sequence of Py-γ TIP, three asparagine residues with potential N-glycosilation sites were also present at positions 78, 97, and 168.

Relation between VM23P and Py-γ TIP

VM23 is the tonoplast water channel of radish, and two kinds of cDNA encoding VM23 were cloned, namely γ-VM23 and δ-VM23 (Higuchi et al., 1998). They suggested that the antibody against VM23 was raised against mixture of γ- and δ-VM23, although γ-VM23 was predominant.

Having used the antibody against VM23, we previously reported the developmental change in the protein level of the tonoplast water channel of pear fruit (VM23P, Shiratake et al., 1997a). In this study, we detected two spots of VM23P in immunoblotting after 2D-PAGE of tonoplast proteins (Fig. 3). Their molecular masses were similar, but their pl's were different; i.e., the pl of the major spot was 4.8, whereas that of minor one was 5.2. The calculated pl of a translated product of Py-γ TIP is 4.86. Hence, the spot at pl 4.8 might be translated from Py-γ TIP. The spot at pl 4.8 was stained more densely than that at pl 5.2, not only by immunostaining (Fig. 3) but also by silver staining (data not shown). This indicates that the translated product of Py-γ TIP might accumulate predominantly and play a major role in tonoplast water channels of pear fruit.

The spot at pl 5.2 may represent an isogene, e.g., δ-type TIP, rather than an allele, because the difference in pl between two spots in 2D-PAGE is not close. However, amino acid sequencing of two spots is essential to determine the exact relationship between two spots and Py-γ TIP.

Expression of Py-γ TIP during pear fruit development

When the total RNA fractions, prepared from pear flesh at different stages, were subjected to northern blot analysis (Fig. 4). The results revealed that mRNA levels of Py-γ TIP, based on rRNA content, were especially high at 23 and 41 DAF. The level decreased dramat-
ically at 57 DAF and became trace after 83 DAF.

The levels of γ TIP and its homologues were especially high in the cell elongation and/or cell differentiation zones of Arabidopsis (Ludevid et al., 1992), cauliflower (Barrieu et al., 1998), maize (Chaumont et al., 1998) and tulip (Balk and de Boer, 1999). From these observations, Chrispeels et al. (1999) postulated that the high expression of γ TIP is necessary to sustain vacuole biogenesis and to prepare the cells for the rapid increase in vacuolar volume that accompanies cell enlargement.

For approximately 40 days after flowering, pear fruit undergoes rapid cell division (cell-division stage). After the cell division slows down, the fruit enlarges only by cell expansion (Bain, 1961; Yamaki and Matsuda, 1977). The pear cell at 30 DAF already has a central vacuole, which occupies a large part of the cell volume (Shiratake et al., 1998). Hence, at the cell-division stage, pear fruit grows, not only by cell division, but also by vacuole biogenesis and by its enlargement. The pear fruits at 23 and 41 DAF were at the middle and the end of cell-division stage, respectively. A high expression of Py-γ TIP at this stage (Fig. 4) is consistent with the results in other plant species as stated above. Thus, our finding supports the postulation of Chrispeels et al. (1999), that Py-γ TIP sustains vacuole biogenesis and enlargement.

An expression of γ TIP was increased by gibberellin (GA) in Arabidopsis plant (Phillips and Hutty, 1994), which may explain why the high expression of γ TIP occurs in young tissues. In general, concentration of GA in the flesh of fruit is relatively high before and/or just after flowering, and the application of exogenous GA to young fruit increases fruit size. Perhaps, the transcript level of γ TIP is also regulated by endogenous GA, which would explain their relationship.

Further investigations

During the pear fruit development, VM23P (protein) level is especially high in young fruit and remains relatively high until 76–104 DAF (Shiratake et al., 1997a). Py-γ TIP (mRNA) level, which is also high in young fruit, becomes very low at 57 DAF (Fig. 3). Why Py-γ TIP level differed from VM23P level is attributed to their difference in the means of their expression, that is, Py-γ TIP level is expressed on the basis of rRNA, whereas, VM23P level is expressed on a fresh weight basis or on the basis of total protein. Secondly, there are isogenes of Py-γ TIP, e.g., δ-type TIP, which may regulate VM23P level with Py-γ TIP. These isogenes need to be isolated to prove this latter point in order to establish a clear-cut relationship between two VM23Ps separated by 2D-PAGE and the Py-γ TIP.

Acknowledgments

The authors thank Dr. Masayoshi Maeshima of Nagoya University for the kind gift of antibody against radish VM23.

Literature Cited


セイヨウナシ果実の液胞膜水チャンネルcDNAの単離とその発現
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摘 要

セイヨウナシ果実から液胞膜の水チャンネルをコードするcDNAを単離した。そのクローンの推定アミノ酸配列は、シロイヌナズナのγ-TIP, δ-TIPおよびα-TIPとそれぞれ77%, 52%および50%の同一性を示し、そのクローンがγ-TIP型の液胞膜水チャンネルに属するものと考えられ、Py-γ-TIPと名付けた。また、Py-γ-TIPの推定アミノ酸配列には、水チャンネルに特徵的な6回の膜貫通領域と2カ所のNPAモチーフが存在した。ダイコンの液胞膜水チャンネルであるVM23に対する抗体は、セイヨウナシ果実から単離した液胞膜タンパク質の二次元電気泳動上に、pI 4.8のメジャーなスポットとpI 5.2のマイナーなスポットを検出した。Py-γ-TIPの翻訳産物の推定pIが4.86であることから、メジャーなスポットの方がPy-γ-TIPの翻訳産物だと予想され、Py-γ-TIPが果実中で優位に機能している可能性が示唆された。果実の生長過程におけるノーザン解析の結果は、Py-γ-TIPの発現が果実の細胞分裂期の集中期（開花後23日目と41日目）に特に高いことを示した。この結果から、Py-γ-TIPは液胞の形成過程やその後の液胞の急速な肥大生長に備えて、細胞分裂期に多く発現している可能性が示唆された。

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