Isolation and Expression Analysis of a Gene Encoding a Vacuolar-type Water Channel Protein in Peach Fruit

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Summary

A cDNA clone, Pr-gTIP1, encoding a water channel protein was isolated from the mesocarp tissue of peach fruits (*Prunus persica* Batsch). The deduced amino acid sequence of the cDNA was highly homologous to those of tonoplast intrinsic proteins (TIPs) isolated from other plants; it contained two NPA (asparagine, proline and alanine) motifs, that are part of the pore structure of water channels. Genome gel DNA blot analysis indicates that the gene exists in the peach genome as a single copy. RNA gel blot analysis reveals that Pr-gTIP1 is expressed at an early and late stages of fruit development.

Key Words: aquaporin, fruit, peach, *Prunus persica*, tonoplast intrinsic protein.

Introduction

Water absorption is very important for the growth and development of a fruit. Rapid enlargement of the fruit is induced by increased water absorption to cause cell expansion. Excess absorption of water during fruit development, however, results in a) decrease in sugar concentration and b) fruit cracking reducing fruit quality. Thus, while regulation of water transport is necessary for fruit growth and quality, its control mechanisms have not yet been clarified.

Recently, water channel proteins named aquaporins have been isolated and characterized as molecules for controlling the water permeability of biomembranes in animals and plants (Maurel, 1997; Kjellbon et al., 1999). These water channels form a large family and has been classified into two types of localization in the cell. The Tonoplast Intrinsic Protein (TIP) is located in the vacuolar membrane; the Plasma membrane Intrinsic Protein (PIP) is located in the plasma membrane. Genes that encode aquaporins from several plants have been isolated and characterized. The expression pattern of the aquaporins in plants depends on the genes (Weig et al., 1997) and their functions, such as cell division, cell elongation, cell enlargement (Ludevid et al., 1992) and osmoregulation (Kjellbon et al., 1999). The plants of *Arabidopsis thaliana*, to which the antisense PIP1 aquaporin gene was introduced, developed abnormal root systems, suggesting that aquaporins play an important role in water transport (Kaldenhoff et al., 1996).

Shiratake et al. (1997) identified a 23 kDa antigen in pear fruits that was recognized by an antiserum raised

against TIP from radish (Maeshima, 1992). Their data indicate that the amount of antigen, VM23P, is especially high during the active cell-expansion stage in young fruits which suggests that VM23P might play an important role in the rapid expansion of cells as a vacuolar water channel. However, more information about aquaporins in fruits and the genes encoding them is needed to clarify the mechanisms of fruit growth.

To understand the molecular mechanisms of water absorption by fruits and the functions of aquaporins in these processes, we isolated a cDNA encoding aquaporin from peach fruits. The expression of the gene during peach fruit development was investigated.

Materials and Methods

Plant materials

Peach fruits (*Prunus persica* Batsch cv. Akatsuki) from the orchard of the Agricultural and Forestry Research Center of the University of Tsukuba were harvested in 1998 and stored at –80 °C until used.

cDNA cloning

Total RNA was isolated from mesocarp tissues of peach fruits 60DAFB by phenol/SDS method. The first strand cDNA was synthesized with oligoT17–NotI primer and total RNA. Primer A (5′–GGN GGN CAY GTI AAY CCI GCI GCI GT–3′) and primer B (5′–ARI GGI CCI RCC CAR SAN AYC CA–3′) were used for the first PCR. Nested-PCR was carried out for amplification of TIPs with primer A, primer C (5′–ATR TTI GCI CCI ACD ATR AAN CC–3′) and products of the first PCR. The 300 bp fragment was cloned to pCR2.1 and sequenced. To isolate the full-length cDNA, 5′–RACE and 3′–RACE were performed with specific

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primers for the clone.

DNA gel blot analysis

Genomic DNA was extracted from young leaves of peach and digested with restriction enzymes. Products were fractionated by electrophoresis on a 0.7% agarose gel and fragments were transferred to a Hybond-N⁺ (Amersham). The probe was labeled with the DIG high-prime DNA labeling/detection kit II (Roshe diagnostics). The membrane was hybridized with the probe at 42 °C overnight. The membrane was washed once with 2 x SSC containing 0.1% SDS for 15 min at 25 °C and washed once with a solution of 0.1 x SSC, 0.1% SDS for 30 min at 65 °C. The substrate for alkaline-phosphate reaction was CSPDTM (Roshe diagnostics).

RNA gel blot analysis

Total RNA was isolated by the phenol/SDS method and separated by electrophoresis on 1.2% formaldehyde-agarose gel. The RNA was transferred to Hybond-N⁺ (Amersham) and cross-linked. Hybridization, washing, and detection were performed as described above. The substrate for alkaline-phosphate reaction was CDP-StarTM (Roshe diagnostics).

Results and Discussion

To isolate the cDNA encoding aquaporin gene, the degenerated primers A and B were designed based on the conserved amino acid sequences among aquaporins from animals and plants. The reverse transcriptase-polymerase chain reaction (RT-PCR) was performed with the primers and first strand cDNA, which was synthesized from total RNA of peach fruit tissues. The results of the PCR revealed that about 450 bp fragments were specifically amplified. Nested-PCR was carried out with primer C which was designed based on the conserved amino acid sequences among TIPs and the products of the first PCR as its template. The length of the amplified fragment was estimated to be about 300 bp which corresponds with the lengths of γ -TIPs derived from animals and plants. The sequence of the fragment revealed that the fragments contained a γ -TIP gene. Then, a full length γ -TIP cDNA was isolated by using 5'RACE and 3'RACE. Sequence analysis was performed and the clone was designated as Pr (*Prunus persica*)-g (gamma)TIP1.

When the nucleotide sequences of Pr-gTIP1 were compared with the database of GenBank and EMBL, it was highly homologous to those of γ -TIPs isolated from several plants, such as pears (accession number: AB048248) and *A. thaliana*. In particular, the sequence of Pr-gTIP1 is highly homologous to that of pears in not only the open reading frames but also in the 5' and 3'-flanking region. The Pr-gTIP1 cDNA contains a 756-bp open reading frame and encodes a protein of 252 amino acid (Fig. 1).

When the deduced amino acid sequence of Pr-gTIP1

was compared with those of TIP proteins reported in other plants, they were found to be homologous, especially with that from pears (89%). The clone has two NPA motifs (Asn-Pro-Ala) that are known to be an important motif for the pore structure of the water channel and is conserved among aquaporins (Maurel, 1997).

Genomic DNA gel blot analysis revealed that Pr-gTIP1 specifically hybridized with a single band (Fig. 2). The result indicates that Pr-gTIP1 is encoded by a single gene in the peach genome.

RNA blot hybridization analysis performed to investigate the expression of Pr-gTIP1 during the development of peach fruit (Fig. 3) showed that Pr-gTIP1 was expressed at 14DAFB and again at 98DAFB. Using promoter- β -glucuronidase (GUS) fusions and *in situ* hybridization, Ludevid et al. (1992) found that the expression of γ -TIP gene of *A. thaliana* is correlated with cell enlargement. In Shiratake's work (1997), the protein level of VM23P, the protein which is recognized by antiserum against γ -TIP from radish, was especially high during the period of active cell-expansion in pear fruits. Our experiments indicate Pr-gTIP1 gene was also expressed during the early stage of cell expansion of the peach fruit. Pr-gTIP1 was expressed in the late stage of fruit development when the fresh-weight of the fruit increased rapidly (Fig. 3). At this stage, water absorption is the primary force for cell expansion of

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ATCACTATACTGAGCCAAGAGTGAAGTAGTTTGTGAGGACCGGTACGCAGCTTAATTCTG 60
CAGCGAATCTCCACCGTCGCATCTTAATCATCTCCGACGATGCCGATCCGGAACATTGC 120
      M P I R N I A
CGTTGGCCGACCCGAGGAGGCGTACCACCCGAGCGCTTCGGGGCGGGTCTCGCCGAGTT 180
V G R P E E A Y H P D A L R A G L A E F
CATCTCCACGCTGATCTTCGTCTTCGCCGCGGAGGGCTCCGGCATGGCCTTCGCCAAGCT 240
I S T L I F V F A G E G S G M A F A K L
CACCCACGGGGCGCGACCAACGCCAGCGCCCTCGTCGCCCGCGCTAGCCACCGGATT 300
T H G A A T T P A G L V A A L A H G F
CGGCTCTTCGTGCGCGTCTCCGTCAGTGCAGAACATCTCCGGCGGACACGTCAACCCGCG 360
G L F V A V S V S A N I S G G H V N P A
GGTCACTTTCGGCGCCTTCGTGCGCGCAATATCTCCCTCCCGCGCATCTCTATTG 420
V T F G A F V G G N I S L A L R G I L Y W
GATCGCCAGCTCTCGGGTCCGCGTCCGCGTGCCTCTCAGGTTCGCAACCCAACTA 480
I A Q L L G S A V A C G L L R F A P N Y
CCAGACTACCTCAGCATTTGCTCTCTCCGAGGGGGTTGGGTATGGAACGCGTCTGTTT 540
Q T T S A F A L S E G V G V W N A F V F
CGAGATCGTGATGACGTTTGGGTTGGGTGTACACGGTGTAGCCACAGCCATTGACCCGAA 600
E I V M T F G L V Y T V A I D A P K
GAGAGGCGAGCGTGGGAACAATCGCGCGATCGCGATCGGTTTCTAGTTGGCGCGAAT 660
R G S V G T I A P I A I G F V V G A N I
TTTGGCGGGTGGGCTTTCGATGGAGCATCCATGAACCCGCGCTTCATCTGGGCGCGC 720
L A G G A F D G A S M N P A V S F G P A
TTTGGTGAGCTGGAACGGCACAACCACTGGATCTACTGGCGCGGCGCTTTGATCTGGCGG 780
L V S W N W H N H W I Y W A G P L I G G
TGGGCTTGCTGGGCTTATTACGAGTTCGTCTTCATCGGCAACTCTAGCCACGAGCCACT 840
G L A G L I Y E F V F I G N S S H E P L
CCCGACCGCTGATTACTAATTAATTAAGCTTTAATTAAGCCCTAATTAAGATTCAATCAA 900
P T A D Y *
ATGATATGCATGCATGATGAATCTTCACTTGTGTTTTATGAGGGTTTTGTACCTTTGAATT 960
CTTGTGTTTTATTTCATCAAGAAAGATCATGATCGTTTCATCATGAGTTCGTTGCCACCGT 1020
TGATTGTATTGTTTCGCGATTTCATGCTCCGGTGTATGATTAAATTTAATTTATCCTT 1080
TTTATTATTATAAATTTCTGTAATTTATTTCTTGAAAAA
AAA 1143

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Fig. 1. The nucleotide sequence of Pr-gTIP1. The deduced amino acid sequence shows 2 NPA motifs which are underlined. Asterisk indicates terminal codon.

peach fruit. Thus, the high expression of Pr-gTIP1 is likely involved in cell expansion at this stage.

Since water absorption in fruits is central to several physiological processes in fruits, including enlargement, ripening, and quality, an understanding of the regulatory mechanisms of water flow into fruits is fundamental to understand the basis of fruit growth and development. We have isolated other types of cDNAs encoding TIPs and several cDNAs encoding PIPs from peach fruits

(unpublished). Further analysis is necessary to clarify the functions of these genes and regulatory mechanisms of water absorption in fruits.

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モモ果実からの水チャンネルをコードする cDNA の単離と発現解析

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摘 要

モモ (*Prunus persica* Batsch) 果実の果肉より RT-PCR を用いて水チャンネルタンパク質をコードする cDNA を単離した。推定されるアミノ酸配列は他の植物で報告されている tonoplast intrinsic proteins (TIPs) と高い相同性を示し、水透過に重要とされる NPA(Asn, Pro, Ala1) モチーフを有していた。本遺伝子はモモ果実の発育初期、および後期で非常に強く発現していた。

Fig. 2. DNA gel blot hybridization of Pr-gTIP1. B: *Bam*HI, E: *Eco*RI and H: *Hind*III. The probe (Pr-gTIP1) was labeled with DIG.

Fig. 3. Gene expression of Pr-gTIP1 during fruit development. A: RNA gel blot hybridization; B: ethidium bromide staining of the gel; C: growth curves of peach fruits based on the longitudinal diameter (●) and fresh weight (■) (1998 data). Five micrograms of total RNA isolated from each stage were separated on a gel. The membrane was hybridized with a probe (Pr-gTIP1) labeled with DIG. Lane1, 14DAFB; lane2, 28DAFB; lane3, 64DAFB; lane4, 77DAFB; lane5, 91DAFB and lane6, 98DAFB at harvest. Arrows indicate the date of sampling for expression analysis.