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# Identification and cloning of *PIP1* gene in carrizo citrange (*Citrus sinensis* × *Citrus trifoliate*)

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**Abstract**

The genus *Citrus* (Rutaceae) is economically very important and is grown in tropical and subtropical areas of the world. One of the most important factors in raisingthe quantity and quality of citrus production is sufficient water. Aquaporinsare integral membrane pore proteins and conduct water molecules in and out of the cell. Plant aquaporins divided into four subgroups including plasma membrane intrinsic proteins (PIPs), tonoplast membraneintrinsic proteins (TIPs), nodulin-26–like intrinsic proteins (NIPs) and small basic intrinsicproteins (SIPs). The present study was carried out for identification of PIP1 gene in Carrizo citrange (*Citrus sinensis*×*Citrus trifoliate*). From results, a fragment with 867- bais-pair (bp) lengthwith high similarity to PIP1gene in plants was identified as a probable member of the PIP gene family. The cloned cDNA sequence has been submitted to Gen Bank under the accession number KJ546461.1.This gene is encoding a deduced protein containing 288 amino acids. The three-dimensional structural model of the protein was also constructed by SWISS-MODEL server, indicating that the gene structure of PIPs has been highly conserved.

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**Introduction**

The necessity of water for plants is completely clear. It has many functions in the plant, and continuously absorbed from the soil by roots and lose out from stomatal pores through transpiration stream (Johansson *et al.*, 2000). Long-distance water transport is carried out in the vascular tissues by xylem and phloem. In this way water transport is by bulk flow which does not present significant membrane barriers. In contrast, short-distance transport and non-vascular tissues requires water transfer across living tissues and cell membranes. There are three ways for water movement in plant tissues, known as; apoplastic, symplastic and transcellular pathways(Javot *et al.*, 2003).

Structurally, the apoplast is formed by the continuum of adjacent cell walls as well as the extracellular spaces. In the symplastic pathway, water is transported in the cytoplasmic continuum of adjacent cells connected via plasmodesmata. In the trans cellular pathways, water is transported across cell membrane(Johansson *et al.* 2000). In the later case water passes mainly through plasma membranes by two routes: by diffusion through the lipid bilayer and through water channels called aquaporins(Javot and Maurel, 2002).Aquaporins are proteinaceous pores in cell membranes that facilitate and regulate the passive diffusion of water and small neutral solutes across biological membranes of most living cells(Agre *et al.*, 1998; Aroca *et al.*, 2006).Some eventssuch as transpiration or expansion growthrequire intense flows of water in living tissues. Therefore, water movement through lipid bilayer of cell membrane cannot be responsible for such fluxes. Aquaporins play an important role in rapid transmembrane water flow (Johansson *et al.*, 2000).

Aquaporins are small integral membrane proteins belonging to the ancient family of major intrinsic proteins (MIPs)(Maurel *et al.*, 2008).In most plant species, aquaporins can be divided into four subgroups. The plasma membrane intrinsic proteins (PIP) (with two phylogenic subgroups, PIP1 and PIP2, and 13 isoforms in Arabidopsis) and the tonoplast intrinsic proteins (TIP) (10 homologs in Arabidopsis) are the most abundant aquaporins in plant cells (Johanson *et al.*, 2001; Sakurai *et al.*, 2005).The third subfamily includes the nodulin-26–like intrinsic membrane proteins (NIPs),which werenamedafter soybean (Glycinemax) nodulin-26 (GmNOD26), an abundant aquaporin expressed in the peribacteroid membrane of N2-fixing symbiotic root nodules. NIPs are also present in non-legume plant species (9 homologs in Arabidopsis)(Wallace *et al.* 2006). A fourth class comprises small basic intrinsicproteins (SIPs) (3 homologs in Arabidopsis)(Ishikawa *et al.*, 2005). Although these four classes are conserved among all plant species, the aquaporin gene family shows signs of rapid and recent evolution and orthologs cannot necessarily be distinguished between species(Maurel *et al.*, 2008). Aquaporins are likely to be important for water transport both at the cellular and at the whole plant level, but the full signific ance of aquaporins in plant water relations remains unclear(Hachez *et al.*, 2006).

Plant aquaporins show a high multiplicity of isoforms. For instance, by study of whole genome sequences 35 aquaporin genes in *Arabidopsis thaliana*(Quigley *et al.*, 2002),33 in*Oryza sativa* L.(Sakurai *et al.*, 2005), 28 in *Vitis vinifera* L.(Fouquet *et al.*, 2008)and 23 in a moss called *Physcomitrella patens*(Danielson and Johanson, 2008) were identified.

The water movement through aquaporin is driven by osmotic or pressure gradients, i.e. aquaporins are not active pumps or transporters. They are a simple pores that allows a bidirectional flow of water across membranes due to water potential differences(Maurel *et al.*, 2008).The plant aquaporins have high sensitivity to a wide range of environmental stresses. Accordingly, aquaporin genes can be regulated at the transcriptional level in response towater stress conditions such as osmotic, drought, salt, and cold stress or abscisic acid (ABA) treatments(Boursiac *et al.*, 2005; Bray, 2001).

The genus Citrus (Rutaceae) is economically very important and is grown in tropical and subtropical areas of the world. (Cheng and Roose, 1995; Agustí *et al.*, 2014). Citrus species are characterized by abundant foliar development and a large canopy. Consequently, they tend to have fairly high water requirements. (Cohen *et al.*, 1997). Therefore, fruit qualityandyield is reduced bylong-term water stress. Drought stress also can delayfruit growth and increase fruit abscission(Arbona *et al.*, 2005;García-Sánchez *et al.*, 2007).

Regarding the importance of aquaporins in plants, it is essentialto identify the members of MIP gene family in major crops. As mentioned before, the presence ofa number of aquaporins genes were reported in some important plant speciessuch as*Arabidopsisthaliana*, *Oryza sativa* and*Vitis vinifera*. In the present work, the genes encoding putative aquaporins in the *Poncirus trifoliata* genome were identified. The results obtained provide a platform for further studies on aquaporins in the other members of *Citrus*.

**Material and methods**

*Plant Materials and sampling*

This study was carried out in the agricultural research station of Tabriz University. Experiment was accomplished using one year old plants oftrifoliate orange (*Poncirus trifoliate*)that were purchased and transplanted into 30-L plastic pots filled with a mixture of peat moss and perlite (1:1).The potted plants were kept in greenhouse condition under natural photoperiod throughout the experiment. The average day/night temperature was 33/21˚C and relative humidity varied diurnally from 38% to 86%. Prior to onset of main experiment, prepared plants were fed weekly with a dilute solution of a complete fertilizer (Plant prod 20-20-20, Plant Products Co. Ltd., Ontario Canada) supplemented with 6% iron chelate in a sufficient volume to leach from the bottom of all pots. Generally, media water content were adjusted and kept in field capacity (FC).Root samples were collected from the apical half of the roots. Samples packed in aluminum foil then were frozen in liquid nitrogen and stored at -80˚C until RNA extraction.

*RNA extraction and synthesis of the first-strand cDNA*

Frozen roots were ground to powder in liquid nitrogen and RNA extraction was carried out by using RNX-Plus™ (Sinaclon, Karaj, Iran) RNA extraction solution. Evaluation of RNA extraction qualitywas done by electrophoresis and spectrophotometry methods.All RNAsamples were treated with RNase-free DNase I (Sinaclon) for degradation of DNA.For RT–PCR, the first strand cDNA was synthesized by using RevertAid™ MMuLV Reverse Transcriptase (Fermentas Inc.) according to kit protocols.

*PCR amplification*

Forward and reverse primersweredesigned based on PIP1 gene conserved sequence regionsin the other plants available in GenBank and using by Oligo7 software. Primers were synthesized as follows: sense (ATGGGGAAGGATGTTGAAGT) and antisense (TTAAGCATTGCTCCTGAAGG) (TAG Copenhagen, Denmark).A suitablereaction condition for each primer set was determined using genomic DNA prior to the actual reaction. Amplification of aquaporin genes was performed by using a DNA thermocycler (Eppendorf). The PCR was conductedin the following profile: Initial denaturation at 94°C for 5 min followed by 94°C for 1 min, 53°C for 90 sec, 72°C for 1 min for 30 cycles and final elongation at 72°C for 5 min. The obtained cDNA was subjected to agarose gel electrophoresis and stained with res safe.

*Transformation*

Resulting PCR products were ligated into a pTZ57R/T vector in the presence of T4-Ligase enzymeusing the procedures described by the manufacturer's instructions (Fermentas Inc.). Then, the ligated products were transformed into *Escherichia coli*s train DH5α competent cells with TSS method(Chung *et al.*, 1989). Transformed cells were platedat 37 °C in

LB broth for 45 minutes and subsequently were selected on LB-Amp (100 µg/mL) plate supplemented with IPTG and X-Gal. Screening of target colonies was conductedby colony PCR method withPIP1 gene specific primers andpositive colonies were cultured in liquid medium. Finally, after obtaining the appropriate concentration, plasmid extraction was performed using purification protocol of the plasmid extraction kit (Fermentas Inc.).

*Sequence analysis*

In this study, the following programs and databases were used: BLAST and Gen bank at NCBI, Prosite at the ExPASy Server and ClustalW at EBI server and three-dimensional model of PIP1 in Swiss Model server. Genes encoding putative aquaporins were identified at the National Center for Biotechnological Information (http://www.ncbi.nlm.nih.gov).Analysis of nucleotide sequences of PIP1 genewere carried out using MWG automatic DNA Sequencing Service (TAG Copenhagen, Denmark) and comparison with known sequences available in the Gen Bank database using BLAST analysis (Altschul *et al.*, 1990).

**Results and discussion**

*Molecular cloning of full-length cDNAs and characterization of PIP1*

Oligonucleotide primers designed from highly conserved regions of the available plant aquaporins wereused to screen of PIP1 gene in Carrizo citrange.

This led to the identification of fragment with 867-bais-pair(bp) length (Fig. 1).The cloned cDNA sequence has been submitted to Gen Bank under the accession number KJ546461.1. Nucleotide sequence of PIP1 gene was obtained using MWG automatic DNA Sequencing Service (TAG Copenhagen, Denmark) (Fig. 2).

Based on BLAST analysis in NCBI database, Carrizo citrange putative aquaporin PIP1 mRNA sequence indicated high similarity (99%) with *Citrus sinensis* PIP1-2-like (LOC102616421). According to the high similarity of PIP1in Carrizo citrange and other species, we conclude that PIP1 is a member of the PIP gene family. This geneis encoding a deduced protein containing 288 amino acids.

**Table 1.** List of Domain hits.

| NAME | ACCESSION | DESCRIPTION | INTERVAL |
|---|---|---|---|
| MIP | cd00333 | Major intrinsic protein (MIP) superfamily. | 54-279 |
| MIP | Pfam00230 | Major intrinsic protein MIP, (Major Intrinsic Protein). properties. | 46-276 |
| MIP | TIGR00861 | MIP family channel proteins. | 58-276 |
| GlpF | COG0580 | Glycerol uptake facilitator and related aquaporins (Major Intrinsic Protein Family). | 57-280 |
| PLN00027 | PLN00027 | aquaporin TIP; Provisional | 54-279 |
| PRK05420 | PRK05420 | aquaporin Z; Provisional | 87-279 |
| PTZ00016 | PTZ00016 | aquaglyceroporin; Provisional | 53-271 |

*Protein structure and conserved domain analysis*

The search for the conserved domain in putative aquaporin PIP1 protein in NCBI Conserved Domain Database showed that PIP1 contains two important domains, explicitly GlpF (Glycerol uptake facilitator and related aquaporins) and MIP (Major intrinsic protein superfamily) (Fig. 3).

The list of domain hits of putative aquaporin PIP1 proteinis shown in Table 1. Furthermore, the motifsof PIP1 in Carrizo citrange and some other plant species are similar. These results suggest that the predicted amino acid sequence of PIP1 is relatively conserved. The three-dimensional structural model was also constructed by SWISS-MODEL (Fig. 4).

*Homologous alignment and phylogenetic analysis of PIP1gene*

Through the alignments of nucleotide sequences, a phylogenetic tree was constructed to determine evolutionary relationship among PIP from Carrizo

citrange and other species. As shown in Fig. 5, Carrizo citrange has the closest relationship with *Citrus sinens*is and Citromelo, and relatively close relationship with *Citrus clemantin*, *Iris xhollandica*, *Eucalyptus grandis*and *Arabidopsis thaliana*. However, PIP1 showed a close relationship with all the above PIPs, indicating that the gene structure of PIPs has been highly conserved.

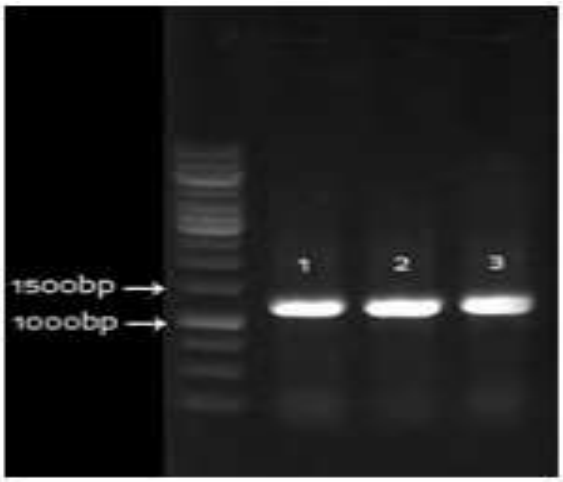


**Fig. 1.** PCR product of PIP1 gene on 1% agarose gel electrophoresis.

The molecular bases of water transport in citrus have not been studied.

Many genes encoding PIP super family were isolated from different plants but up to now no genes have been identified from Citrus genus. We isolated a PIP2 like sequence from Poncirus trifoliata and Swingle citrumelo.

Amino acid sequence analogy with different plant aquaporins show that these sequences bear motif similarity with that of the PIP2 family. All plant plasma membrane aquaporins, including these sequences, presented two highly conserved regions, one in the loop C: GGGANXXXXGY and other in loop E: TGI/TNPARSL/FGAAI/VI/VF/YN (Barone *et al.* 1997).

In human AQP1 protein four residues (Phe 58, His 182, Cys 191, Arg 197) defined the constriction region of the channel pore, and three of these are conserved across the water-specific aquaporins.

```
ATGGAGGGCAAAGAAGAGGACGTTAGATTAGGAGCCAATAAGTTCAATGAGAGGCAACCGATTGGAACAG
CAGCTCAGAGCCAAGATGGCAAGGACTACGTTGAGCCGCCGCCGGCACCACTCTTCGAGGAAGAAGAGCT
CACGTCTTGGTCGTTTTACAGGGCCGGTATAGCCGAGTTCATAGCCACTTTTCTTTTCTTGTACATCTCC
GTATTGACAGTGATGGGCGTCGTCAAGTCCAACACCAAGTGCTCAACCGTCGGCATTCAAGGAATTGCAT
GGGCCTTCGGTGGCATGATCTTCGCGCTCGTTTACTGCACAGCTGGCATCTCGGGTGGTCATATCAACCC
GGCGGTGACATTCGGGCTGTTCTTGGCGAGGAAGCTGTCGTTGACACGTGCACTTTTCTACATGGTGATG
CAGTGTCTGGGAGCCATCTGCGGTGCTGGCGTTGTAAAGGGTTTCATGGGTGCCGGCCAATACGGGCGGC
TCGGCGGCGGTGCCAATGCTGTAGCTCATGGCTACACCAAGGGTGATGGTCTTGGTGCCGAGATTATTGG
TACTTTTGTTCTTGTCTACACAGTTTTCTCTGCCACTGATGCCAAGCGTAGCGCAAGAGACTCCCATGTT
CCTATTTTGGCTCCTCTGCCAATTGGGTTCGCTGTGTTCTTGGTTCACTTGGCTACTATCCCTATTACCG
GAACTGGCATCAACCCCGCAAGGAGTCTTGGAGCTGCAATCATCTACAACAAGGACCACGGTTGGGATGA
CCATTGGATTTTCTGGGTGGGACCATTCATTGGAGCAGCACTTGCAGCTTTGTACCACCAGGTTGTGATC
AGAGCCATTCCCTTCAAATCAAGCTGA
```

**Fig. 2.** Nucleotide sequence mRNA, complete cds of PIP1 gene in Carrizo citrange.

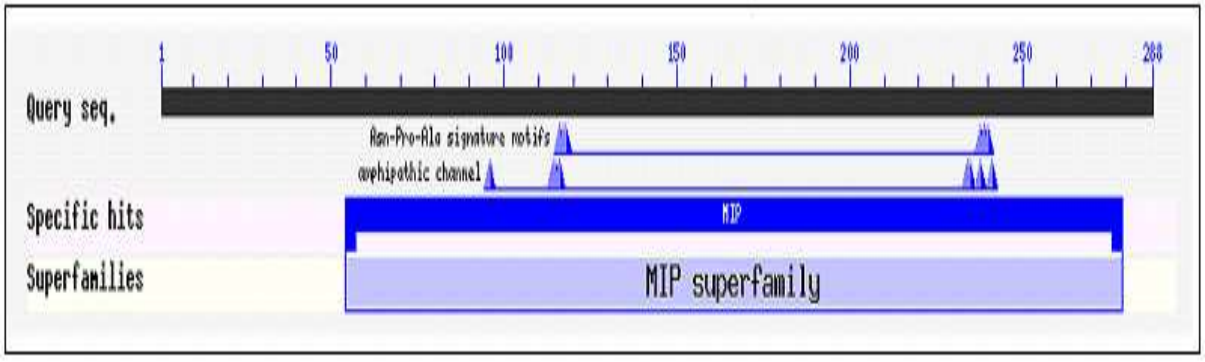


**Fig. 3.** Putative conserved domains of PIP1 aquaporin protein.

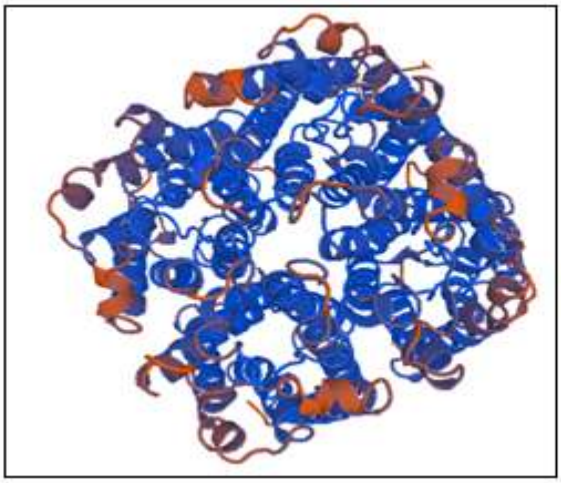

**Fig. 4.** Predicted PIP1 three-dimensional model.

The location of these residues is essential for defining the selectivity to water or to others solutes as glycerol (Sui *et al.* 2001). The activity of plasma membrane aquaporins may be affected by cytosolic pH. A conserved His residue in the intracellular loop D seems to control this effect, as shown by the reduced effects of cytosol acidification when this amino acid is substituted by an alanine (Tournaire-Roux *et al.* 2003). Both olive aquaporins contain this His residue (OePIP1.1- His207, OePIP2.1-His201), suggesting that also these olive proteins may sense pH (secchi *et al.* 2007).

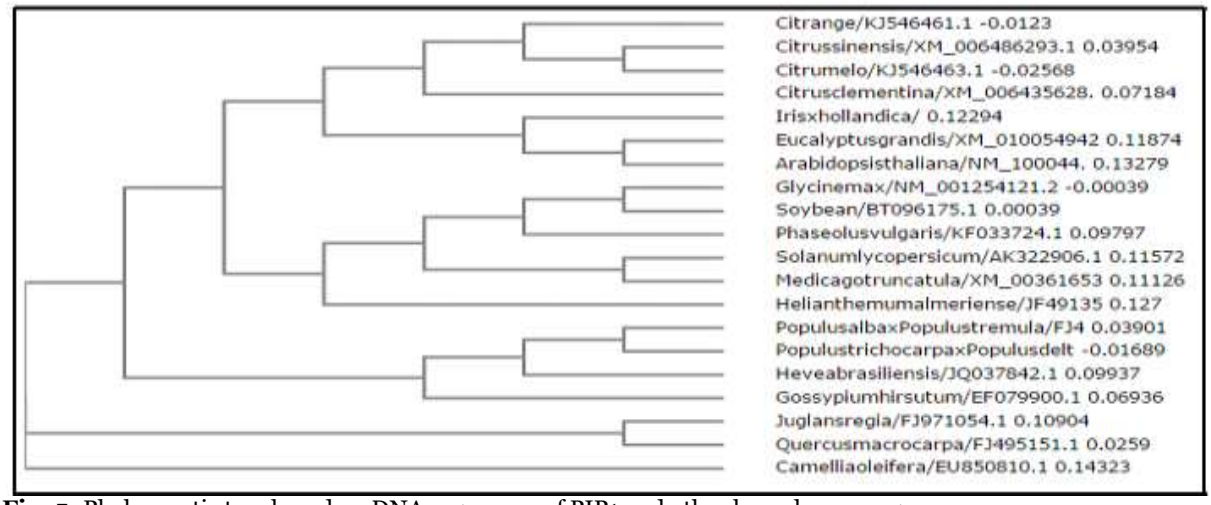


**Fig. 5.** Phylogenetic tree based on DNA sequences of PIP1 and other homologues sequences.

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