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Identification and cloning of PIP2-like gene in *Poncirus trifoliata* and *Swingle citrumelo*

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Abstract

Water transport through cellular membranes is facilitating by water channels, the so-called aquaporins. Aquaporins were shown to be involved in many physiological processes such as root water uptake, reproduction or photosynthesis. There are some evidences confirming that PIP proteins could regulate the whole water transport through plant tissues. This study is aimed to isolate the PIP2 gene in *Poncirus trifoliata* and Swingle citromelo. The fragment of this gene was amplified by polymerase chain reaction and then was cloned in a TA vector. The sequence of PIP2 gene exhibited a high similarity with the other plant PIP2 genes in the NCBI gene bank. The results indicate that this gene sequence owns a higher identity (97%) with *Citrus sinensis* PIP2-1-like gene that belongs to the same taxonomic family with trifoliate orange (Rutaceae).

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Introduction

The genus Citrus, belonging to the rue family (Rutaceae), encompasses most important tree fruit crops in the world. However, citrus production is affected by adverse environmental conditions such as drought and salinity (Bañuls and Primo-Millo 1995, Gong and Liu 2013). In particular, exposure to these stresses challenges the plant water status and trigger specific strategies for cell osmotic adjustment and control of water uptake (Boursiac *et al.* 2005, Hasegawa *et al.* 2000). Water uptake and transport by roots were facilitated by aquaporins, which selectively conduct water molecules (Tyerman *et al.* 2002).

In the whole plant, long-distance water transport occurs mostly through vascular tissues, therefore membrane barriers are not restrictive (Kaldenhoff *et al.* 2008). However, in short-distances water can flow different pathways: the apoplastic, symplastic and transcellular paths (Steudle and Frensch 1996). Thus, presence of aquaporins in the membranes of living cells can extensively increase the water permeability (Kaldenhoff *et al.* 2008, Maurel *et al.* 2008).

Aquaporins belongs to the major intrinsic proteins (MIP) family with many homologous genes (Maurel *et al.* 2008). So far, 14 aquaporins were identified in mammals (King *et al.* 2004) whereas plants exhibit a higher abundance of aquaporin homologs (Kaldenhoff and Fischer 2006). In plants, aquaporins are subdivided into four major groups (Kaldenhoff and Fischer 2006): plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), NOD26-like intrinsic proteins (NLMs, NIPs) and small basic intrinsic proteins (SIPs). The PIP subfamily can be further subdivided into two groups, i.e. PIP1 and PIP2 (Johanson *et al.* 2001).

In general, aquaporins are essential for many biological processes in plants such as root water uptake, cell elongation, leaf movement, movement of guard cells, flowering and fertilization (Martre *et al.* 2002, Kaldenhoff *et al.* 2008). It has been suggested that aquaporin expression was responsive to water stress in *Vitis* hybrid which resulted in constant leaf water potential and hydraulic conductivity (Galmes *et al.* 2007). In olive, a negative relationship between root growth and aquaporin expression is reported. This means that in roots of the olive dwarf clones, expression of PIP1 and PIP2 was higher than in roots of vigorous clones (Lovisolo *et al.* 2007).

Despite the importance of PIP genes in plants, the sequence of these genes has been remained unclear in citrus. Therefore, the aim of the present study was to isolate and identify of PIP2 gene in two important rootstocks of citrus.

Materials and methods

Plant Materials

Plant materials of two citrus rootstocks, i.e., trifoliate orange (*Poncirus trifoliata*) and Swingle citrumelo (*Citrus paradisi* × *Poncirus trifoliata*) was obtained from Agricultural Research Center of Amol city. The plants were transplanted into 30-L plastic pots filled with a peat moss, perlite (1:1) soilless media. The potted plants were kept in greenhouse condition under natural photoperiods.

RNA isolation and first strand cDNA synthesis

Total RNA was extracted from root samples of plants using the RNX-Plus™ extraction buffer (Cinnagen, Iran). Quality and quantity of total RNA were determined by agarose gel electrophoresis and spectrophotometry.

To generate first-strand cDNA, one microgram of the total RNA was used as a template in a 20 µl reaction mixture containing 15 pmol of gene-specific primer and the nuclease free water up to 12.5 µl. This solution was first denaturated at 65°C for 5 minutes and chilled on ice immediately. Then, the mixture of 20 U ribonuclease inhibitor, 1 mM dNTPs, the 5x buffer supplied by the manufacturer and 200 U RevertAid™ MMuLV Reverse Transcriptase (Fermentas Inc.) was added to the reaction. Ultimately, Reaction mixture was incubated at 42°C

for 60 minutes and at 70°C for 10 minutes afterwards.

PCR amplification

A pair of gene-specific primers was designed based on PIP2 gene sequence in the GenBank using Oligo7 software. Primers were synthesized by as follows: sense (ATGGGGAAGGATGTTGAAGT) and antisense (TTAAGCATTGCTCCTGAAGG) (TAG Copenhagen, Denmark).

The synthesized cDNA was amplified by PCR using specific primers of PIP2 gene. The cycling conditions were as follows: initial denaturation at 94°C for 5 minutes followed by 35 cycles of 94°C for 1 minutes, 54°C for 1 minutes, 72°C for 2 minutes, and a final extension of 72°C for 5 minutes. The amplified fragments were analyzed on 1% agarose gel electrophoresis.

Transformation, Screening and Colony PCR

The PCR products were cloned into pTZ57R/T vector using the procedures described by the manufacturer's instructions (Fermentas Inc.).The ligated products were then transformed into the *E. coli* strain DH5a at which competent with TSS method (Chung *et al.* 1989). Transformants were grown at 37 °C in LB broth for 45 minutes, Then were selected on LB-Amp (100 µg/mL) plate supplemented with IPTG and X-Gal. Recombination confirmed by colony PCR with PIP2 gene specific primers. Finally, the recombinant plasmids were extracted from white colonies according to the purification protocol of the plasmid extraction kit (Fermentas Inc.).

Sequence and analysis

In this study, the following programs and databases were used: BLAST and Genbank at NCBI, Prosite at the ExPASy Server and ClustalW at EBI server. The nucleotide sequences of the PIP2 gene were determined using MWG automatic DNA Sequencing Service (TAG Copenhagen, Denmark) and compared with other sequences available in the GenBank database using BLAST analysis (Altschul *et al.* 1990).

Results and discussion

PCR amplification and nucleotide sequencing

Using the primers for amplification of PIP2 gene the length of fragments was determined as 879 bp and 738 bp in trifoliate orange and Swingle citrumelo, respectively (Fig. 1).

Table 1. List of Domain hits.

Name	Accession	interval
MIP	cd00333	46-276
MIP	pfam00230	38-273
Glycerol_uptake_facilitator protein	TIGR00861	50-273
GlpF	COG0580	41-277
PLN00166	PLN00166	46-273
PRK05420	PRK05420	84-277
PTZ00016	PTZ00016	30-277

The sequences are submitted to Genbank under different accession numbers (*Poncirus trifoliate*/ KJ546467.1; *Citrus paradisi x Citrus trifoliate*/ KJ546464.1). According to BLAST analysis of sequence, the higher identity (97%) for *Poncirus trifoliata* putative aquaporin PIP2 mRNA was obtained with *Citrus sinensis* PIP2-1-like (LOC102627699) that belongs to the same taxonomic family with trifoliate orange (Rutaceae).

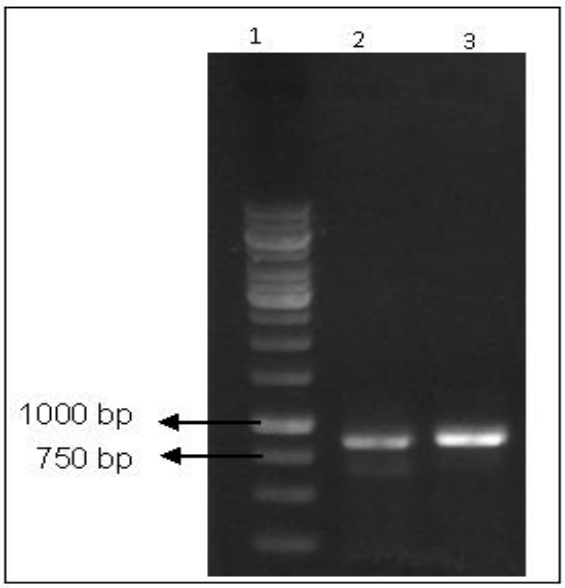

Fig. 1. PCR product of PIP2 gene on 1% agarose gel electrophoresis. Lane 1, size marker; lane 2, Swingle citrumelo and lane 3, trifoliate orange.

Phylogenetic and motif analysis

Phylogenetic analysis using DNA sequence data showed a high relationship to each of known sequences of the PIP gene (Fig. 3). *Citrus clemantin* showed close relationship with Citromelo and trifoliate orange.

The molecular bases of water transport in citrus have not been studied. Many genes encoding PIP superfamily 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.

Fig. 2. Nucleotide sequence of mRNA, complete cds of PIP2 gene A) trifoliate orange b) Swingle citrumelo.

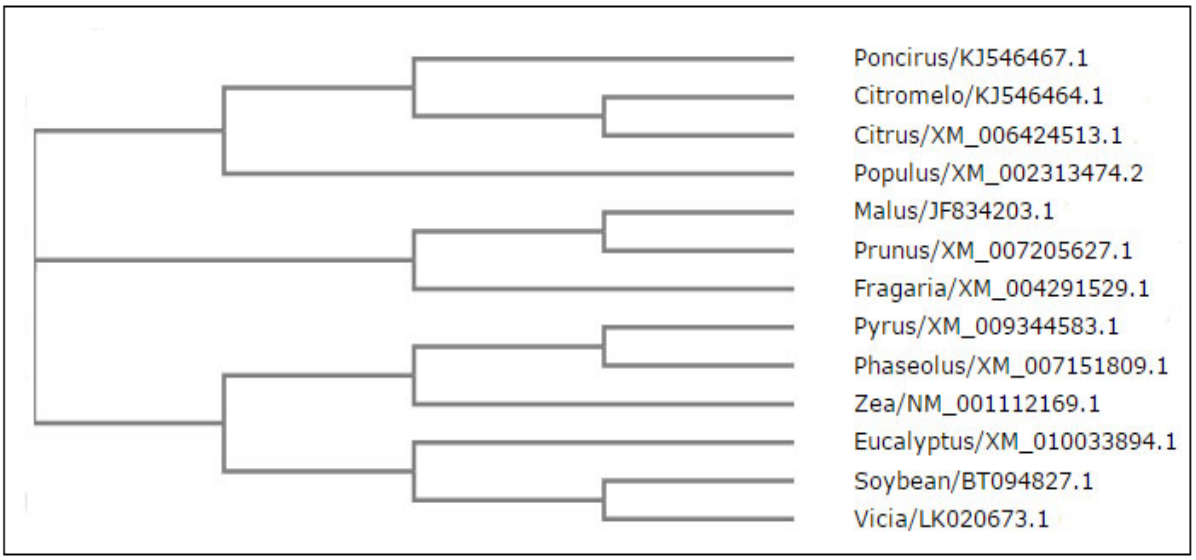

Fig. 3. A phylogenetic tree for aligned DNA sequences of the PIP gene.

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).

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