

Full Length Research Paper

Molecular cloning and characterization of the plasma membrane aquaporin gene (*OvPIP*) from *Orychophragmus violaceus*

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A plasma membrane aquaporin gene (*OvPIP*) from *Orychophragmus violaceus* (*O. violaceus*) was cloned. The full-length cDNA of *O. violaceus* gene (*OvPIP*) was 1314 bp and contained 1188 open reading frame encoding a protein of 395 amino acids. Homology analysis revealed that *OvPIP* strongly resembled other *PIP* genes. Southern blot analysis indicated that *OvPIP* gene was present in *O. violaceus* genome, and quantitative real-time PCR (QRT-PCR) analysis revealed that *OvPIP* transcript level was most abundant after 12 h treatment with 200 mM sodium chloride. Our studies suggested that *OvPIP* is a member of the family of the recently cloned plant plasma membrane aquaporins gene.

Key words: *Orychophragmus violaceus*, plasma membrane, tonoplast aquaporins gene, RACE-PCR, quantitative real-time PCR.

INTRODUCTION

Aquaporins (AQPs) belong to the family of major intrinsic proteins, which facilitate the transport of water and other small neutral molecules across cell membranes (Johanson et al., 2001). In plants, AQPs form a large family with 35 members in *Arabidopsis* (Johanson et al., 2001). Based on the sequence similarity, the higher plant AQP family can be classified into four subfamilies: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin 26-like intrinsic proteins (NIPs) and small basic intrinsic proteins (SIPs) (Chaumont et al., 2001; Johanson and Gustavsson, 2002; Johanson et al., 2001). Recent studies have identified XIPs in the primitive plant *Physcomitrella patens*. Members of the fifth subfamily, XIPs, are uncharacterized and are absent in *Arabidopsis*,

rice and maize (Gupta and Sankararamakrishnan, 2009). The PIP subfamily is further divided into two subgroups named PIP1 and PIP2 that have specific arrays of amino acids at the N and C-termini (Schafer, 1998) whereas, several PIP2 isoforms (AtPIP2;1, AtPIP2;2, AtPIP2;3, AtPIP2;7; Kammerloher et al., 1994; Daniels et al., 1994; Weig et al., 1997) have been reported to function as water channels, and the substrate specificity of PIP1 isoforms remain less clear. The large number of AQPs found in plants suggests their importance during plant development and adaptation to the changing environment. Many processes in plants are dependent on massive water flow into and out of the cells, and therefore it is reasonable that AQPs function in several cellular processes, including the movement of leaves, cell elongation, response to stress and sexual reproduction (Chaumont et al. 1998; Moshelion et al., 2002; Jang et al., 2004; Bots et al., 2005). They function as cellular plumbers in maintaining not only the ubiquitous water transport from the roots to shoots but also cell homeostasis at all developmental stages and under all environmental conditions.

Soil salinity is a prevalent abiotic stress for plants.

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Abbreviations: QRT-PCR, Quantitative real-time PCR; AQPs, aquaporins; PIPs, plasma membrane intrinsic protein; MIP, major intrinsic protein; *O. violaceus*, *Orychophragmus violaceus*.

Excess salts in the soil solution would interfere with mineral nutrition and water uptake, and lead to undue accumulation of toxic ions (Hasegawa et al., 2000). Plant growth under salt stress depends on the re-establishment of proper cellular ion homeostasis with other concomitant processes. Low cytosolic Na⁺ content was preserved by the concerted interplay of regulated ion uptake, vacuolar compartmentation and active extrusion to the extracellular milieu (Blumwald et al., 2000). Jang (2004) reported that marked up- or down-regulation in PIP expression was observed by drought stress, whereas PIP genes were less-severely modulated by high salinity.

Orychophragmus violaceus belongs to *Orychophragmus*, *Brassica* in Cruciferae, which is mainly distributed in China. It has been found that *O. violaceus* subjected to salt stress of 150 mmol L⁻¹ NaCl solutes displays strong tolerance and normally grows flowers and seeds when planted in field, but *Arabidopsis* belongs to Cruciferae which only grows in 70 mmol L⁻¹ NaCl solutes. Up till now, there has been no report on the molecular cloning of *OvPIP* gene from *O. violaceus*. In this study, the molecular cloning and characterization of the *OvPIP* gene from *O. violaceus* was reported.

MATERIALS AND METHODS

Plant material

The seeds of *O. violaceus* were sterilized in 5% sodium hypochlorite solution for 10 min followed by 3 rinses with sterile distilled water and then were sown randomly in thoroughly washed sand in plastic containers (40 × 30 × 6cm³) with drainage holes in the bottom. The seedlings were irrigated on alternative days with full-strength Hoagland nutrient solution. After 8 days, the seedlings were transplanted into bigger plastic pots (22.5 cm in diameter and 22.5 cm in depth) and continued irrigating with the same fresh nutrient solution. 29 days later, 200 mM NaCl was added into the nutrient solution. Total RNA was extracted from young leaves of the plant treated with 200 mM NaCl for 24 h and was used for the cloning of *OvPIP* cDNA.

Cloning and sequencing of *OvPIP* full-length cDNA from *O. violaceus*

Total RNA was extracted using TRIZOL Reagent (GIBCO BRL, USA) according to the manufacturer's instructions. An aliquot of the isolated 150 ng RNA was reverse transcribed with a cDNA synthesis primer AP (5'-GGCCACGCGTCTCGACTAGTAC(T)16-3'). According to the conservative sequence of *PIP* from *Arabidopsis thaliana*, a pair of primers, primer 1 (5'-GGAGAAGGGTTTCAGACAAG-3') and primer 2 (5'-CGAGAGACA AGGAACCTGAA-3'), were designed and synthesized, and PCR was carried out in a total volume of 50 µl containing 2 µl cDNA, 10 pmol each of primer 1 and primer 2, 10 µmol dNTPs, 1 × cDNA reaction buffer and 5 U *Taq* polymerase. The PCR reaction was performed under the following conditions: the template was denatured at 94°C for 2 min followed by 35 cycles of amplification (94°C for 1 min, 55°C for 1 min and 72°C for 1 min) and by 10 min at 72°C. The PCR product (*PIP1*, 800 bp) was purified and cloned into a pGEM-T vector (Promega, USA), and then sequenced. Blast search revealed that the PCR product had a high degree of similarity with two cDNA sequences from *Brassica napus*

plasma membrane intrinsic protein 1 (*PIP1*) mRNA and *A. thaliana* plasma membrane intrinsic protein 2A. Primer 3 (5'-ATTTGCGGTG TTYATGGTGCACCT-3') was subsequently designed and synthesized according to the *PIP1* sequence. The 3' RACE of *OvPIP* full-length cDNA was performed with the 3' RACE system for rapid amplification of cDNA ends (RACE, GIBCO BRL, USA) in a total volume of 50 µl containing 2 µl cDNA, 10 pmol each of primer 3 and AP primer, 10 µmol dNTPs, 1 × cDNA reaction buffer and 5 U *Taq* polymerase. The PCR reaction conditions were the same as those described earlier. The PCR product (*PIP13'*, 387 bp) was purified and cloned into a pGEM-T vector, and then sequenced. Based on the *PIP1* sequence, complementary reverse gene specific primers, primer 4 (5'-AGGAAGAGGAGAGTAGC-3') and primer 5 (5'-CTTGTCTGAAACCCTT CTCC-3'), were designed to amplify the 5' end of *PIP1* cDNA. RNA was reversely transcribed with primer AP, followed by tailing cDNA with oligo (C). The first round of PCR was performed with primer 4 and Abridged Anchor Primer (AAP, 5'-GGCCACGCGTCTCGACTAGTACGGGIIIGGGIIIGGGIIIG-3') under the following conditions: the template (2 µl cDNA) was denatured at 94°C for 3 min, followed by 35 cycles of amplification (94°C for 1 min, 56°C for 1 min and 72°C for 2 min) and by 7 min at 72°C. The PCR product was diluted 50-fold for the second round of amplification with primer 5 and AUAP (5'-GGCCACGCGTCTCGACTAGTAC-3') under the same PCR conditions. The PCR product (*PIP 5'*, 338 bp) was purified and cloned into pGEM-T vector and then sequenced. The earlier mentioned three fragments were assembled into a full-length cDNA (*OvPIP*) through alignment and deletion of the overlapping fragment with VNTI 6.0 software. Based on the full-length cDNA sequence, gene specific primers *OvPIP* F1 (5'-GCAATAAAAAAGTATACAAC-3') and *OvPIP* R1 (5'-CAGTAAAA GCATGGATATTAT--3') were used for the amplification of the coding region fragment of *OvPIP* by RT-PCR following the manual described by the manufacturer (TAKARA RT-PCR kit) and the amplified sequence of *OvPIP* was inserted into pGEM-T vector (Promega) followed by sequencing.

Southern blot analysis

Southern blot was carried out to analyze the copy number of *OvPIP* present in *O. violaceus* genome. For Southern blot analysis, genomic DNA was extracted from young leaves of the seed that germinated from *O. violaceus* seedling (23 days-old) by cetyl trimethyl ammonium bromide (CTAB) method as described before (Doyle and Doyle, 1990). Aliquots of genomic DNA (8 mg) were digested overnight at 37°C with *Xho I* and *Bam HI*, respectively, fractionated by 0.8% agarose gel electrophoresis and transferred to a Hybond-Nt membrane (Amersham). The part of the *OvPIP* cDNA coding fragment from 324 to 1042 bp (718 bp) was used as the probe and there were no *EcoRI*, *Xho I* and *Bam HI* restriction sites in this 718 bp probe sequence. Hybridization bands were detected using the DIG Luminescent detection kit (Roche), and signals were visualized by exposure to Fuji X-ray film at 37°C for 15 min.

The expression profile of *OvPIP* under salt stress

To investigate the expression pattern of *OvPIP* under salt stress, total RNA was extracted from the *O. violaceus* plants treated with various concentrations of NaCl (0, 100, 150, 200 and 250 mM NaCl) for various durations (0, 6, 12 and 24 h) and was used for the QRT-PCR analysis. RNase-free DNaseI (Takara, Japan) was used to digest the genomic DNA in the total RNA preparation. 100 µg of total RNA was then synthesized into the 1st strand cDNA using a Reverse-transcription kit (Takara, Japan). QRT-PCR reactions were carried out in a PTC-200 RT-PCR system (Bio-Rad) in MJ white PCR tubes according to the manual of two-step QuantiTect SYBR Green PCR Kit (Qiagen #204143). The total PCR volume was 25 µl

at aaaaaagt at acaaacaaat t aacaagaaggagagct ct cact
 at aaaacagagcacat gt t ct t ct cact aaccact gaaact agt t
 94 at ggcgaaggacgt ggaagccgt t t ccggagaagggt t t cagaca
 M A K D V E A V S G E G F Q T
 139 agagact at caagat ccaccgccagcgct ct gt t t gacccggcg
 R D Y Q D P P P A P L F D P A
 184 gagct gaccaagt ggt ct t t ct acagagcagt cat cgct gact t c
 E L T K W S F Y R A V I A E F
 229 gt agct act ct cct ct t cct gt acat caccgt t t t gacagt cat c
 V A T L L F L Y I T V L T V I
 274 ggt t acaagat t cagaccgat agt act gccggcggcgt t gact gc
 G Y K I Q T D S T A G G V D C
 319 ggt ggggagaagggt t t cagacaagagact at caagat ccaccgc
 G G E K G F R Q E T I K I H R
 364 cagcgcct ct gt t t gaccggcgagcgt ggccaagt ggt ct t t ct
 Q R L C L T R R S W P S G L S
 409 acagagccagt cat cgct gact t cgt agct act ct cct ct t cct g
 T E P V I A E F V A T L L F L
 454 cacat cacct gt t t ggaycagt cr at cgggt t at ccaagat t caga
 H I T C L D Q S I G Y P R F R
 499 ccgat agt act gccggcggcgt t gact gcggt ggagt t ggt at cc
 P I V L P A A L T A V E L V S
 544 t t gt at cgct t gggct t t t ggt ggcgt gat ct t cat cct t gt ct a
 L Y R L G F W W H D L H P C L
 589 ct gcaccgcccgt at ct ct ggt ggt cacat t t accggcagc gac
 L H R R Y L W W S H L P G S D
 634 at t t gggct act ct t ggcgcgt t aagt gt cct t ggt agagccat
 I W A T L G A L S V L G K S H
 679 gt t gt acat ggt agct cagt gt t t ggggt gcgat at gt ggagt t gg
 V V H G S S V F G C D M W S W
 724 gt t cgt caaagcct t ccaagact ct t act acgt ccgt t acggt gg
 V R Q S L P K L L L R P L R W
 769 t ggagccaact ct ct agccgacgggt acagcacaggaaccggact
 W S Q L S S R R L Q H R N R T
 814 agccgcagagat cat t ggt act t t cgt t ct t gt ct at accgt ct t
 S R R D H W Y F R S C L Y R L
 859 ct ccgccact ggcct t aacgt at t gccagagact cccacgt ccc
 L R H W P L T Y C Q R L P R P
 904 agt gt t ggcgccact t cccat t ggat t t gcggt gt t cat ggt gca
 S V G A T S H W I C G V H G A
 949 ct t ggct accat t cccat cact ggaact ggaat caaccggcaag
 L G Y H S H H W N W N Q P G K
 994 gact t t cggagcagccgt t at ct t caacgagagcaagccat ggga
 E F R S S R Y L Q R E Q A M G
 1039 t gccact ggat at t t t ggggt gggaccat t cgt t ggagct gcgat
 W P L D I L G G T I R W S C D
 1084 agct gcat t ct accaccagt t cgt t t t gagggt t caggt t ccaa
 S C I L P P V R F E G F R F Q
 1129 gt ct ct cggagt t t gat cct at gt agat t aaaggt t ct t t caaga
 V S R S L I L C R L K V L S R
 1174 caagagcct t t ggt aaat accct t t t gt at t t t caagcact t t cc
 Q E P L V N T L L Y F Q A L S
 1219 t t t ct t cagt gt gt t ct t t t t t aggt t gt t agt ct ct t ct
 F L Q C V F F S F R L L V S S
 1264 act caat gt at t cat taa 1281
 T Q C I H *
 aaaaaaat t at at aat at ccat gct t t t act c

Figure 1. The full-length cDNA sequence and deduced amino acid sequence of *OvPIP* gene. The start codon (atg) is underlined and the stop codon (taa) is underlined and it is in italics.

and the amplification program was used: 95°C for 10 min; 45 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, 76°C for 2 s (checking fluorescence change); followed by 55 to 94°C to check the amplification specificity. Primers were designed with Primer 12 Express 2.0 (Applied Biosystems) for PCR products that were 100 ~ 150 bp in length (*OvPIP* QF15'—GAGCTGACCAAGTGGTCTTTCTAC-3', *OvPIP* QR15'—GCATCAACGCCGCCGGCAGTACTA-3').

Standard curves were then determined using eight serial 10-fold dilutions of the earlier known plasmids concentration. The expressed copies of the test genes and internal control (actin) were calculated according to their respective standard curve.

RESULTS

Isolation and analysis of *OvPIP* full-length cDNA of *O. violaceu*

Using the conserved sequence of the *PIP1* gene from *A. thaliana* as an internal fragment, 813-bp fragment was obtained from cDNA of the young leaf of *O. violaceu* treated with 200 mM NaCl for 24 h via RT-PCR. After sequencing the internal fragment, we used 5' and 3' RACE PCR to capture the upward and downward fragments of *OvPIP*. The amplified upward, downward and internal fragments (PIP5', PIP3' and PIPBC) were then assembled to get the full-length cDNA of *OvPIP* by alignment and deletion of overlapping fragments. By using the method described in the paper, the full-length cDNA sequence of *OvPIP* was obtained through RT-PCR reaction and subsequently confirmed by sequencing. The full-length cDNA (GenBank Accession No: bankit1252551) of *OvPIP* was 1314 bp and contained 1188 open reading frame encoding a protein of 395 amino acids, a 5'-untranslated region of 93 bp and a 3'-untranslated region of 33 bp. The predicted *OvPIP* protein was 395 aa in length with a molecular weight of 45806.95 Da and an isoelectric point of 9.58; these predictions were made based on a web analysis program/on web analysis software (<http://us.expasy.org>). The complete nucleotide sequence and the deduced amino acid sequence of *OvPIP* are shown in Figure 1.

Southern blot analysis

Southern blot was carried out by digesting genomic DNA isolated from the leaves with *EcoRI*, *Bam HI* and *Xho I*, respectively to analyze the *OvPIP* present in *O. violaceu* genome. Multiple hybridizing bands were detected by Southern analysis, indicating that the *OvPIP* was present in *O. violaceu* genome (Figure 2).

The expression profile of *OvPIP* under salt stress

QRT-PCR analysis was carried out to establish the expression profile of the *OvPIP* of *O. violaceu* plants under salt stress. The results showed that, *OvPIP* was

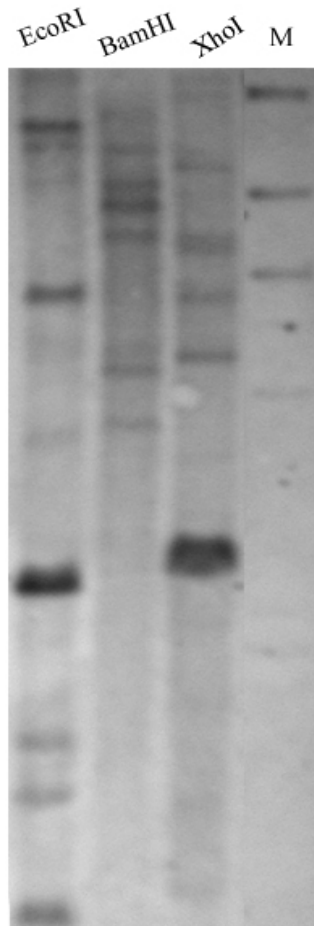


Figure 2. Southern blot analysis of *OvPIP* from *O. violaceus*. Genomic DNA (8 mg) was digested with *EcoRI*, *BamHI* and *XhoI*, respectively, followed by hybridization with DIG Luminescent labeled. *OvPIP* cDNA fragment from 324 to 1042 bp (718 bp) was used as the probe.

expressed at different levels after treated with various concentrations of NaCl (0, 100, 150, 200 and 250 mM NaCl, respectively). There was a little expression of *OvPIP* without salt treatment, while its expression increased under 100 and 150 mmol L⁻¹ NaCl salt treatments, reached its highest level under 200 mmol L⁻¹ NaCl and then began to decrease (Figure 3). On the other hand, we exposed *O. violaceus* plants to 200 mmol L⁻¹ NaCl for 0, 6, 12, 24 and 48 h, respectively. The *OvPIP* gene was expressed at a low level without salt treatment, and its expression increased after the salt treatments, reached its highest level after 12 h salt treatment and then began to decrease (Figure 4).

DISCUSSION

By using the method described in the paper, the full-length cDNA sequence of *OvPIP* was obtained through

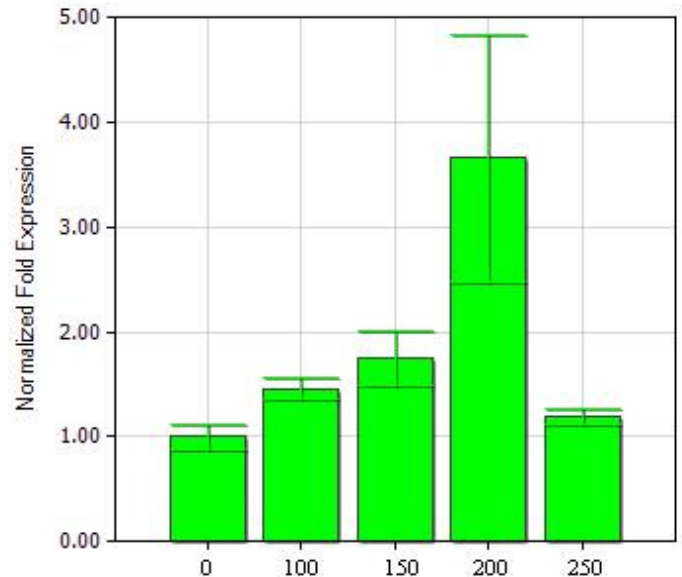


Figure 3. The expression profile of *OvPIP* gene under the various NaCl concentration (0, 100, 150, 200 and 250 mmol L⁻¹) for 24 h determined by quantitative RT-PCR (actin gene as internal standard). Fold inductions are the ratios of the copies of *OvPIP* genes and the actin in *O. violaceus*.

RT-PCR reaction. The full-length cDNA (GenBank Accession No: bankit1252551) of *OvPIP* was 1314 bp and contained 1188 open reading frame encoding a protein of 395 amino acids, a 5'-untranslated region of 93 bp and a 3'-untranslated region of 33 bp. The predicted *OvPIP* protein was 395 aa in length with a molecular weight of 45806.95 Da and an isoelectric point of 9.58; these predictions were made based on a web analysis program/ on web analysis software (<http://us.expasy.org>). The deduced *OvPIP* protein was used in the multiple alignment analysis using the ClustalX program; the results showed that, at the amino acid level, *OvPIP* had 84 and 57% identities, respectively, with the PIP1 from *B. napus* and *A. thaliana*. The phylogenetic analysis suggested that the *OvPIP* protein and the PIP1 of *B. napus* can be classified into one superfamily (Figure 5). Database retrieval with PSI-Blast showed that the putative protein of *OvPIP* belonged to the major intrinsic protein (MIP) superfamily. Members of the MIP superfamily function as membrane channels that selectively transport water, small neutral molecules, and ions out of and between cells. The *OvPIP* proteins share a common fold: the N-terminal cytosolic portion followed by six transmembrane helices, which might have arisen through gene duplication.

Many genes encoding AQPs were isolated from different plant species but up till now, no gene has been identified from *O. violaceus*. In this study, the molecular cloning and characterization of the *OvPIP* gene from *O. violaceus* was reported. The putative protein of *OvPIP* belonged to the PIPs. Southern blot analysis indicated that the *OvPIP* was present in *O. violaceus* genome. To

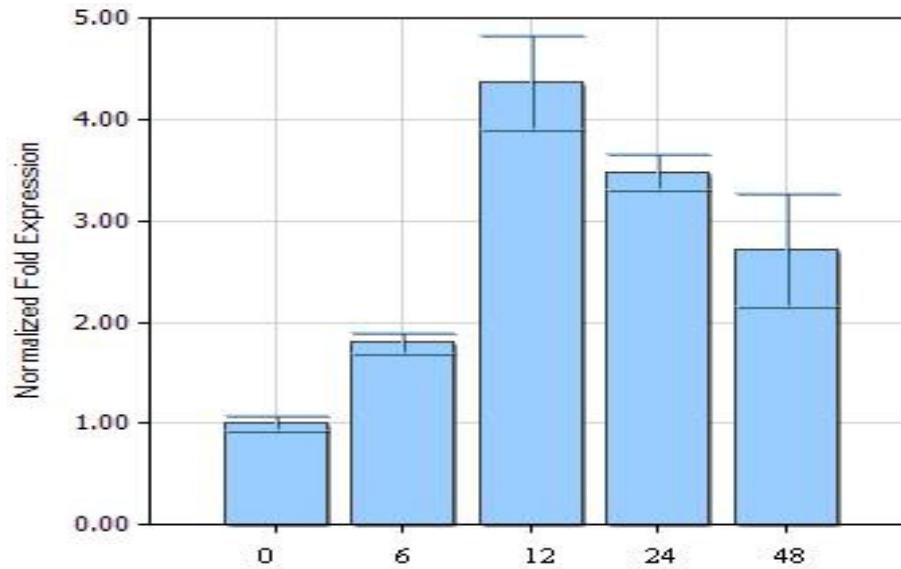


Figure 4. The expression profile of *OvPIP* gene under 200 mmol L⁻¹ NaCl for 0, 6, 12, 24 and 48 h determined by quantitative RT-PCR (actin gene as internal standard). Fold inductions are the ratios of the copies of the *OvPIP* gene to the expression level at 0 mM (or before the treatment). The ratio was further corrected by the actin expression levels.

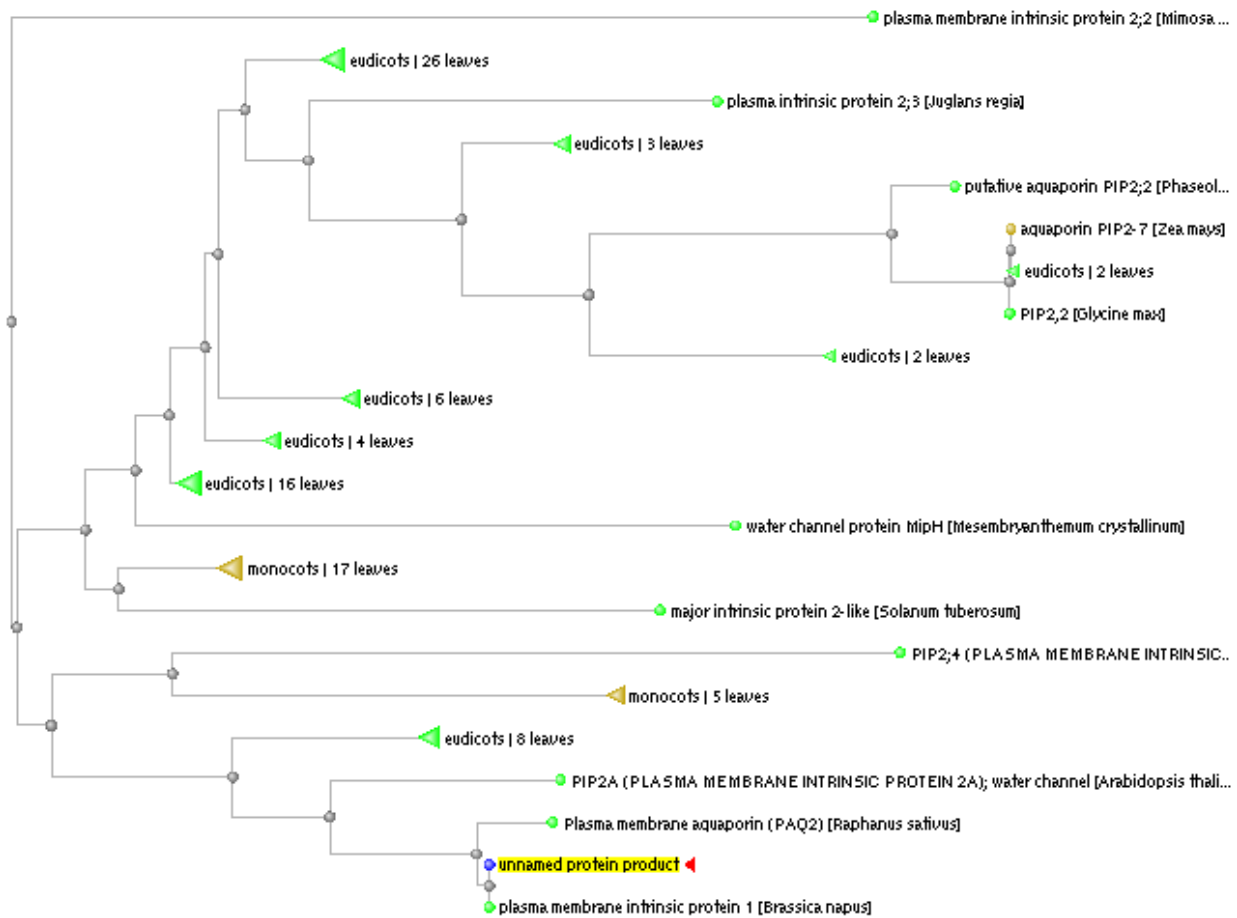


Figure 5. Phylogenetic tree of the *OvPIP* and related protein from other plant. The unnamed protein product is the *OvPIP*.

investigate the effect of high salinity on the transcript level of *OvPIP* gene, the *O. violaceus* plants were subjected to the indicated treatments, and the expression levels of *OvPIP* were measured by QRT-PCR. During the 0 to 200 mM NaCl treatment, the *OvPIP* expression increased but it began to decrease when the concentrations of NaCl reached 250 mM. On the other hand, we exposed *O. violaceus* plants to 200 mM NaCl for 6, 12, 24 and 48 h, respectively, and QRT-PCR analysis revealed that the *OvPIP* expression in leaves could be detected at a low level without salt treatment and reached the highest level after 12 h. Longer NaCl treatment did not result in a larger accumulation of the *OvPIP* transcript. The up-regulation of PIP2; 3 in *Arabidopsis* plants and certain aquaporin isoforms in rice by salt stress were also observed (Liu et al., 1994; Seki et al., 2002). This different down- or up-regulation of aquaporin gene expression during salt stress may play roles in limiting initial water loss during the early stage of salt stress and assisting the subsequent uptake of water to maintain water homeostasis in high cellular salt conditions (Jang et al., 2004).

In conclusion, we reported on the cloning of an aquaporins gene (*OvPIP*) from the leaves of *O. violaceus*. The *OvPIP* was the first aquaporins gene cloned from the leaves of *O. violaceus*. The cloning of the *OvPIP* will enable us to test its potential salt tolerance function by transferring the gene into tobacco in the future.

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