Isolation and expression of an aquaporin-like gene *VfPIP1* in *Vicia faba* *

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Received September 22, 2004; revised October 18, 2004

Abstract To explore the effects of aquaporins on stomatal movement, we isolated a full length cDNA of aquaporin-like gene *VfPIP1* (*Vicia faba* plasma membrane intrinsic protein gene, GenBank accession number: AY667436), which encodes for a 290-amino-acid polypeptide, from *Vicia faba* leaf epidermis by 5′/3′ RACE (Rapid Amplification of cDNA Ends). The analyses of VfPIP1 transmembrane regions and amino acid sequence show that VfPIP1 owns six membrane-spanning domains and the special plasma membrane signature sequences GGGANXXXXGY and TGI/TNPARSL/FGAAI/VI/VF/YN, and it should be a member of PIP1 subfamily. The results of *in situ* hybridization and Northern blot indicate that *VfPIP1* is strongly expressed in guard cells and induced by ABA. Hereby, VfPIP1 may be involved in the water-transmembrane movement of guard cells.

Keywords: ABA, aquaporin, gene cloning, *Vicia faba*, guard cell.

Stomatal pores in the epidermis of plant leaves consist of a pair of guard cells. Stomatal closure or opening influences photosynthesis and transpiration of plants. Water transmembrane into or out of guard cells can lead to turgor change and stomatal movement. Originally, people took it for granted that the water physically passes through the membrane bilayers into or out of cells. However, water-transport rate by the passage is very small. The discovery of plant aquaporins gives a new idea for exploring the regulation of water-transmembrane movement in stomatal movement. So far, over thirty aquaporin genes have been isolated from plants[1,2] and the detailed analyses of the amino acid sequences of the aquaporins have showed that six membrane-spanning domains, two sets of conserved Asn-Pro-Ala motifs and signature sequence of SGXHXNPA are necessary and sufficient for the function of water transport[3,4]. According to the localization and function of aquaporins, the plant MIP superfamily could be divided into four classes: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), NOD26-like membrane intrinsic proteins (NIPs) and small and basic intrinsic proteins (SIPs) subfamily[2,5]. Plants have a higher number of MIPs than mammals. Various studies have revealed the presence of the differences in stage-specific and tissue-specific expressions at the protein levels, protein activities and special functions for plant aquaporins[6—8].

Although many kinds of aquaporin genes have been isolated, most of researches are focused on the aquaporins in roots[9,10]. We have previously detected aquaporins in *Vicia faba* guard cells using the DNA probe and antibodies against RD28 protein[11]. Some other researches have also demonstrated the expressions of aquaporins in *Arabidopsis*, *Vicia faba* and sunflower (*Helianthus annuus*)[12—14]. However, whether there are any kinds of other aquaporins expressed in *Vicia faba* guard cells and how they function in stomatal movement are unclear.

In this study, we isolated a *Vicia faba* guard cell aquaporin-like gene *VfPIP1* from *Vicia faba* leaf epidermis and analyzed its sequence. The expression and localization of this gene were also observed. The results showed that *VfPIP1* was strongly expressed in guard cells and induced by ABA, and VfPIP1 owns the special plasma membrane signature sequences and belongs to the PIP1 subfamily.

1 Materials and methods

1.1 Materials

Seeds of *Vicia faba* were grown in a growth

* Supported by the Major State Basic Research Development Program of China (Grant No. G1999011700) and National Natural Science Foundation of China (Grant No. 30370129)

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chamber with a light/dark photoperiod (12 h/12 h), at a light intensity of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and a day/night temperature cycle of 22 ℃/17 ℃. Total RNA was extracted from 3-week-old leaf epidermis using Trizol reagent (GIBCO-BRL).

1.2 Isolation of *VfPIP1* cDNA

1.2.1 Cloning of *VfPIP1* EST sequence

cDNA was synthesized using oligo $(dT)_{18}$ as a primer and M-MLV reverse transcriptase (GIBCO-BRL). Two oligonucleotide degenerate primers (Primer 1 and Primer 2)[15] were designed based on several conserved regions in the plant aquaporin amino acid sequences.

Primer 1: 5′-(AT)GG(TA)CACAT(TC)AAC-CCAGC-3′,

Primer 2: 5′-AG(AG)CT(GA)GC(CGT)GGGTTGATGCC-3′.

VfPIP1 EST sequence was amplified by RT-PCR using the two degenerate primers, and sequenced directly by Bioasia.

1.2.2 Isolation of the full-length *VfPIP1* cDNA

According to the above EST sequence, we designed two specific antisense primers (GSP1 and GSP2) and two specific sense primers (GSP3 and GSP4)

GSP1: 5′-CCACCGCCTTTCGCACCGAAGAAGGC-3′

GSP2: 5′-CTTGTTAGAGACAGTTTCCTCGCC-3′,

GSP3: 5′-TGGAATCTCTGGGGGTCACATAAACCC -3′

GSP4: 5′-GCCACTGATGCCAAACGTAGTGCCAG-3′

Full-length *VfPIP1* cDNAs were elongated using the 5′/3′ RACE kit (ClonTech) following the instructions of the manufacturer, and cloned and sequenced as described previously. In terms of patch-up full cDNA sequence, we designed two specific primers VfP1 and VfP2

VfP1: 5′-ACGCGGGGATAGCATTCATATTC-3′,

VfP2: 5′-GAATAAACACATGATCCAGATCTCTC-3′

to isolate full-length *VfPIP1* cDNA from *Vicia faba* leaf epidermis cDNA using pfu DNA polymerase. Then the reaction products were separated, cloned and sequenced as described previously.

1.3 Northern blot hybridization

Three-week-old seedlings of *Vicia faba* were treated with 100 $\mu mol \cdot L^{-1}$ ABA for 0.5 h, 1 h, 2 h, 3 h and 5 h, respectively, the untreated as control. Total RNA was prepared using Trizol kit (GIBCO-BRL). Total RNA was fractionated on a 1.5% denatured formaldehyde agarose gel according to Tsang's methods[16] and transferred to a Hybond-N membrane (Amersham) following standard blotting techniques[17]. The probe was *VfPIP1* 3′-UTR sequence labeled with ^{32}P by the random-primer labeling kit (TaKaRa). The prehybridization and hybridization were performed at 65 ℃ in church buffer (1% BSA, 0.25 $mol \cdot L^{-1}$ Na_2HPO_4 (pH 7.2), 1 $mmol \cdot L^{-1}$ EDTA, 7% SDS). Hybridized membranes were washed under high-stringency conditions (2XSSC/0.5% SDS, 0.1XSSC/0.1% SDS at 65 ℃ for 30 min, respectively), and then exposed to radiographic film with intensifying screens at −80 ℃ for three days.

1.4 RNA *in situ* hybridization

The *in situ* hybridizations were performed as described previously[18] with some modifications. Three-week-old *Vicia faba* leaves were fixed in FAA solution (50% ethanol, 5% acetic acid, and 3.7% formaldehyde) at room temperature for 6 h with occasional degassing under a vacuum for 15 min. The tissues were sectioned into 10 μm slices. The labeled *VfPIP1* sense- and antisense probes were generated using a digoxigenin RNA-labeling mixture (Boehringer Mannheim). The probes were hybridized to the tissue sections for 20 h at 50 ℃ at a concentration of 200—400 $ng \cdot mL^{-1}$ in 40 mL of hybridization buffer (6X SSC, 3% SDS, 50% formamide, and 100 $\mu g \cdot mL^{-1}$ tRNA). Then, the sections were incubated twice in washing buffer (2X SSC and 50% formamide) at 50 ℃ for 90 min, treated with RNase A (10 $\mu g \cdot mL^{-1}$ in 2X SSC) at 37 ℃ for 30 min and washed at 50 ℃ for 1 h in washing buffer. The sections were incubated in a blocking solution (0.5% TBS buffer) (Boehringer Mannheim) for 1 h, in 1% BSA and 0.3% Triton X-100 in TBS for 30 min and in the same solution containing alkaline phosphatase-conjugated antibodies (Boehringer Mannheim) at a 1/3000 dilution for 90 min. Unbound antibody conjugate was removed in maleic acid buffer (0.1 $mol \cdot L^{-1}$ maleic acid, 0.15 $mol \cdot L^{-1}$ NaCl, pH7.5) and *VfPIP1* transcripts were detected in color-substrate solution (100 $m \cdot mol \cdot L^{-1}$ Tris-HCl (pH9.5), 0.1 $mol \cdot L^{-1}$ NaCl, 50 $mmol \cdot L^{-1}$ $MgCl_2$, 1/50 NBT/BCIP) for 10 h. The photos were taken under a light microscope (Optiphot-2, Nikon).

2 Results

2.1 The characterization of VfPIP1 sequence

A 0.39 kb fragment which contains a sequence

homologous to plant PIP conserved domains was isolated from cDNA prepared from *Vicia faba* leaf epidermis using primer 1 and primer 2. The corresponding full-length cDNAs were recovered by 5′/3′ RACE kit using two antisense primers (GSP1 and GSP2) and two sense primers (GSP3 and GSP4), respectively. Finally, we obtained a full-length *VfPIP1* cDNA from *Vicia faba* leaf epidermis cDNA using pfu DNA polymerase and both of specific primers of VfP1 and VfP2 (Fig. 1(a)). The analysis of the sequence showed that the *VfPIP1* cDNA is 1039 bp in length and contains an open reading frame of 870 bp, flanked by 5′ and 3′ non-coding regions of 47 and 122 bp, respectively. The *VfPIP1* cDNA sequence has been deposited in GenBank (accession number: AY667436). The protein coding 290 amino acids exhibits six predicted transmembrane helices (Fig. 2), MIP family signature sequence SGxHxNPAVT, the special plasma membrane signature sequence GGGANXXXXGY and TGI/TNPARSL/FGAAI/VI/VF/YN (Fig. 1(b)), and a calculated molecular mass of about 30.7 kD. In addition, a putative PKA phosphorylation site and a PKC phosphorylation site are present in VfPIP1 protein sequence (Ser131 and Ser205, respectively) (Fig. 1(b)). VfPIP1 shows very high identity in amino acid sequence to AtPIP1b, CAB93959, NtAQP1 and ZmPIP1-2 from *Arabidopsis*, *Vicia faba*, tobacco (*Nicotiana tabacum*) and maize (*Zea mays*) (84.48%, 84.14%, 81.1% and 87.24%, respectively), and their amino acid sequences in water channel function domains are the same, too. Moreover, their divergences in amino acid sequence are mainly observed at N-terminals (Fig. 3(a)), which is related to subcellular localization. As compared to PIPs isolated from *Arabidopsis*, VfPIP1 has the highest sequence identity at the amino acid level with AtPIP1b (84.48%), and VfPIP1 is 16 amino acids longer than AtPIP2 at N-terminal (Fig. 3(b)). So VfPIP1 belongs to PIP1 subfamily.

Fig. 1. *VfPIP1* cDNA and amino acid sequences. (a) Full length cDNA sequence. (b) The corresponding amino acid sequence. Boxed (SGxHxNPAVT), (GGGANXXXXGY) and (TGI/TNPARSL/FGAAI/VI/VF/YN) represent MIP family signature sequence and the special plasma membrane signature sequence respectively. Boxed (S) represents a putative PKA phosphorylation site (Ser131) and PKC phosphorylation site (Ser205), respectively.

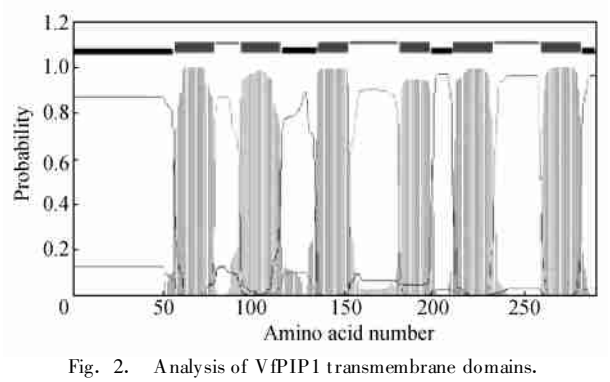

Fig. 2. Analysis of VfPIP1 transmembrane domains.

2.2 Expression patterns of *VfPIP1*

The expression patterns of *VfPIP1* were studied in roots, stems and leaves by Northern blot (Fig. 4 (a)). The results indicated that *VfPIP1* had a higher expression level in roots and leaves than in stems. And the expression of *VfPIP1* gene responsive to ABA indicated that *VfPIP1* mRNA began to increase 1 h after ABA treatment and continuously enhanced with ABA-treatment till 5 h as displayed in Fig. 4 (b). Thus, *VfPIP1* is a gene up-regulated by ABA.

2.3 Localization of *VfPIP1* mRNA

We determined the patterns of *VfPIP1* mRNA in leaf tissues by *in situ* hybridization (Fig. 5). Our results showed that the highest expression of *VfPIP1* was detected in guard cells, and mesophyll cells stained more weakly than guard cells. However, no transcripts were detected in the epidermis cells.

3 Discussion

The discovery of aquaporins in guard cells gives a new idea for studying the mechanism of regulation of stomatal movement. Here, we isolated a *Vicia faba* guard cell aquaporin-like gene *VfPIP1*. Although VfPIP1 water permeability has not been identified, it should be a member of PIP1 subfamily and should have similar functions with PIPs, due to the presence of two conserved NPA motifs, six predicted transmembrane helices, the special plasma membrane signature sequence GGGANXXXXGY, TGI/TNPARSL/FGAAI/VI/VF/YN and amino acid sequence identity in function domains of aquaporins to AtPIP1b, CAB93959, NtAQP1 and ZmPIP1-2.

Our previous study has indicated that aquaporins are present in guard cells and involved in the regulation

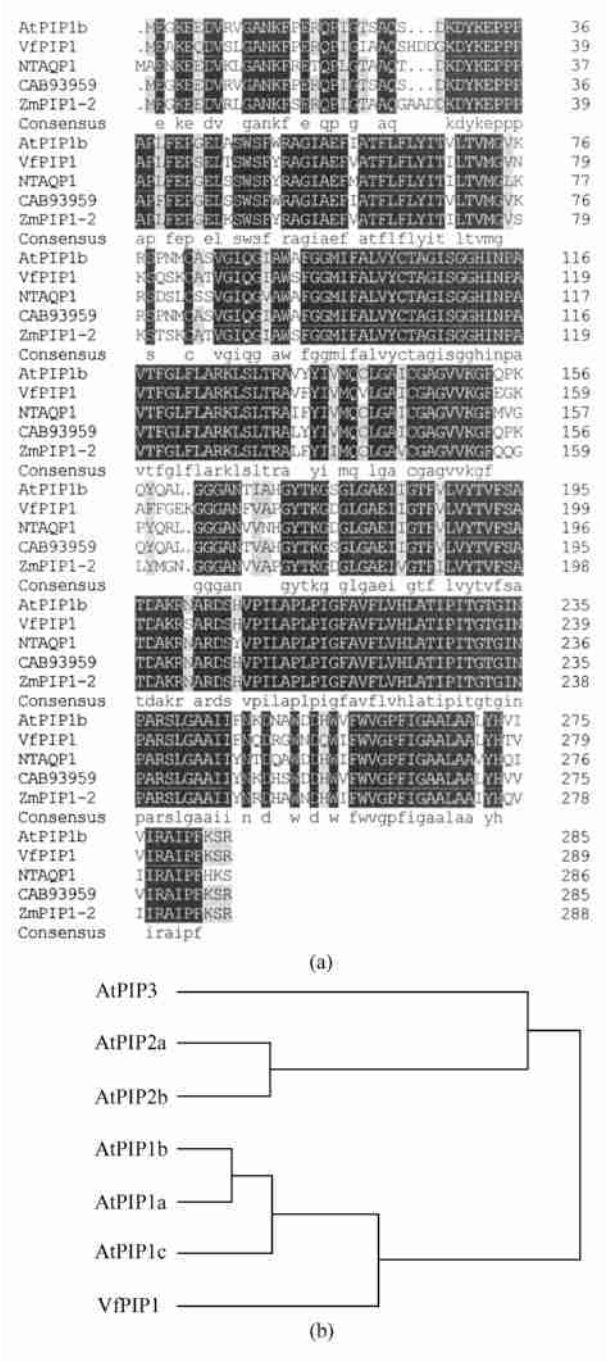

Fig. 3. Comparison of amino acids between VfPIP1 and other plant PIPs (all of sequences from GenBank). (a) Comparison of VfPIP1 sequence with AtPIP1b, CAB93959, NtAQP1 and ZmPIP1-2 from *Arabidopsis*, *Vicia faba*, tobacco and maize. The same amino acid sequences are in black; the divergent amino acid sequences are in grey. (b) The amino acid identity analysis of VfPIP1 and PIPs from *Arabidopsis*.

of stomatal movement[11]. However, the research into aquaporins in guard cells is still insufficient. Kaldenhoff et al.[12] constructed the fusion gene of *AtPIP1b* promoter and GUS (β-glucoronidase gene) and then transformed *Arabidopsis*. They found that

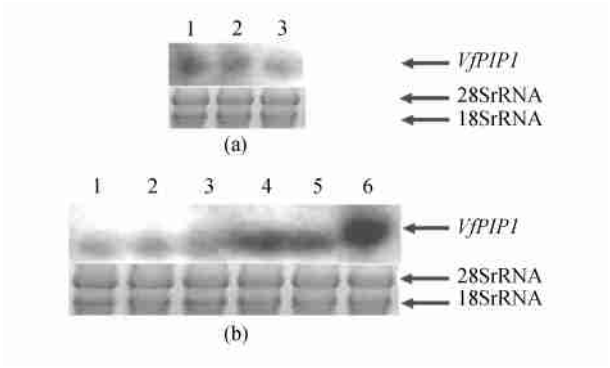

Fig. 4. Northern blot analysis of the *VfPIP1* transcript expression in *Vicia faba*. 28S rRNA and 18S rRNA accumulation is shown below as a control for loading. (a) 1—3 represent root, leaf, stem, respectively; (b) leaves under ABA treatment. 1, control; 2—6 represent 0.5h, 1h, 2h, 3h, 5h after ABA treatment, respectively.

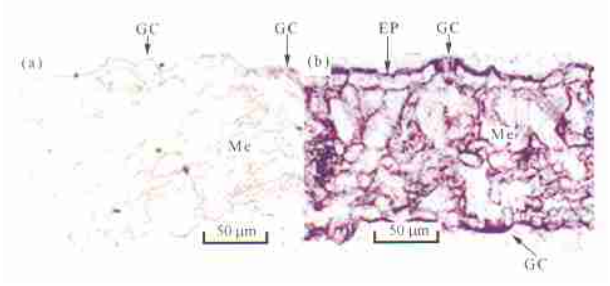

Fig. 5. Localization of *VfPIP1* mRNA in *Vicia faba* leaves by *in situ* hybridization. (a) The control, hybridized with sense RNA; (b) the section hybridized with antisense RNA. GC, guard cells; Me, mesophyll cells; Ep, epidermis cells.

AtPIP1b gene was primarily expressed in expanding as well as in differentiating cells, and GUS activity was also found in guard cells. Sun[13] and Sarda et al.[14] observed mRNA signals of *Vicia faba bbaq1*, sunflower *SunTIP7* and *SunTIP20* in guard cells by *in situ* hybridization. The above evidence further confirmed that aquaporins are present in guard cells. In this study, we isolated a guard cell aquaporin-like gene *VfPIP1* from *Vicia faba*, which acts as a model plant for studying the regulation of stomatal movement. Tissue expression analysis indicated that *VfPIP1* was not exclusively expressed in *Vicia faba*, but more in roots and leaves than in stems. Moreover, the localization in leaves indicated that *VfPIP1* was strongly expressed in guard cells. Hence, VfPIP1 could play an important role in water-transmembrane movement in or out of guard cells.

The regulation of aquaporin activities is a factor for controlling water-transmembrane movement. Many studies showed that plant aquaporins were regulated by various kinds of factors such as environment stimuli, hormones, phosphorylation, Ca^{2+} and pH and each aquaporin owned its regulation way[19—24]. *VfPIP1* gene was induced by ABA. ABA, which is both a product induced by stress signal and a transducer for stress signal, is a very essential stress-responsive hormone in plants, and sustains plant water balance by controlling stomatal aperture[25,26]. Due to the identity of several activated amino acids in loop E to ZmPIP1-2[27], VfPIP1 can function in a similar way with ZmPIP1-2 in regulating water permeability, namely, VfPIP1 is perhaps activated by forming isomers with other aquaporin subunits. In addition, the presence of phosphorylation sites in VfPIP1 sequence could allow an effective modulation of water channel activity. And the previous evidence has showed that both protein kinase and protein phosphorylase are involved in ABA-regulated stomatal movement[28]. Our work indicated that *VfPIP1* gene was expressed strongly in guard cells and induced by ABA. And the analysis of sequence also indicated that VfPIP1 owned the potentially activated amino acid sequence properties. Hence, VfPIP1 may be involved in ABA-regulated stomatal movement by regulating the water-transmembrane movement in guard cells. However, its specific functions need to be further explored using transgenic plants.

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