Identification of Putative Aquaporin Genes and Their Expression Analysis under Hypoxic Conditions in Soybean

[Glycine max (L.) Merr.]

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Abstract: The aims of this study were to detect plasma-membrane intrinsic protein type 2 (PIP2) genes of soybean (cv. Enrei) and to analyze mRNA levels of these genes under normoxic (control) and hypoxic conditions. A sequence similarity search with cDNA sequences in soybean genome revealed three putative aquaporin genes with high homology to GmPIP2;2. The genes were designated GmPIP2 genes. The mRNA levels in roots of soybean seedlings were measured at 3, 6, 12, and 27 hr after hypoxic stress onset. The mRNA levels of all GmPIP2s showed clear diurnal changes in the control, meanwhile, those under hypoxic conditions were significantly down-regulated even at 3 hr after stress onset. These results suggest that the four GmPIP2 genes are transcriptionally co-regulated by the diurnal change and oxygen concentration in the surrounding environment, and the transcriptional regulation may be involved in adjustment of water-transport in roots of soybean seedlings.

Key words: Aquaporin, Gene expression, Plasma membrane intrinsic protein, Root hypoxia, Soybean.

Seeds of the soybean [Glycine max (L.) Merr.] contain a high concentration of oil and protein, and it is one of the major crops cultivated worldwide. Global production of soybean in 2007 was around 219.8 million metric tons (Manavalan et al., 2009). Flooding is one of the abiotic stresses that reduces soybean yield. Sullivan et al. (2001) reported that flooding for as little as 3 d at the early vegetative stage killed soybean plants under the field conditions. Flooding caused by heavy rainfall or over-irrigation reduces oxygen availability around roots and negatively affects plant growth, resulting in drastic yield reduction of soybean (Sallam and Scott, 1987; Griffin and Saxton, 1988; Oosterhuis et al., 1990; Linkermer et al., 1998). Plant roots become stressed during flooding as the rhizosphere becomes hypoxic or even anoxic. One of the earliest responses to these stresses is a reduced water uptake ability of the roots of stressed plants compared with those of well-drained plants (Schildwacht, 1989; Else et al., 1995, 2001). This response is the outcome of decreasing root hydraulic conductance \((L_a)\) or conductivity \((L_p)\), which is often used as an index of the water uptake ability of roots (e.g., Araki, 2006). Araki (2006) demonstrated the reduction of root \(L_a\) in soybean plants subjected to flooding stress for 7 d compared with those of well-drained plants. Tournaire-Roux et al. (2003) and Törnroth-Horsefield et al. (2006) revealed that the cytosolic pH became acid under the anoxic conditions, resulting in closing of aquaporins (AQP) and this led to the decrease of root \(L_p\). AQP s are a family of small pore-forming integral membrane proteins, which contain six membrane-spanning \(\alpha\)-helices, with the N- and C-terminal facing the cytosol (Kruse et al., 2006). Plant AQPs are clearly classified into four subfamilies: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), NOD26-like intrinsic proteins (NIPs) and small basic proteins (SIPs) (Johanson et al., 2001; Zardoya, 2005; Danielson and Johanson, 2008). Some recent researches have also found new three AQP subfamilies, GlpF-like intrinsic proteins...
(GIPs), hybrid intrinsic proteins (HIP) and X intrinsic proteins (XIPs) (Danielson and Johanson, 2008; Gupta and Sankararamakrishnan 2009; Park et al. 2010). The genes within each of the subfamilies can be further divided into subgroups (Zardoya, 2005). Among PIP subfamilies, PIP2s tend to have high water permeability in many plants (Chaumont et al., 2000; Moshelion et al., 2002; Saga and Maeshima, 2004; Fetter et al., 2004; Sakurai et al., 2005, 2008). There are 35 and 39 genes encoding AQPs in the genomes of the model plants Arabidopsis (Johanson et al., 2001) and rice (Bansal and Sankararamakrishnan, 2007), respectively. As for soybean, the only annotated soybean AQP protein sequences in the Genebank are GmPIP1 (accession number; CAI79102; Maitra and Cushman, 1994; Fleurat-Lessard et al., 2005; Porcel et al., 2006), GmPIP2 (accession number; CAI79103; Fleurat-Lessard et al., 2005; Porcel et al., 2006) and GmPIP2;2 (accession number; AAX86046; Klink et al., 2005). Although information on GmPIP2;2 (i.e., sequences of protein and cDNA) is complete, those on GmPIP1 and GmPIP2 are only partial. Recently, nine soybean NIP genes have been identified by Liu et al. (2009). However, there is less information on the AQPs in soybean than those in other plant species. Although Porcel et al. (2006) analyzed the relation between PIP gene expressions and water relations in soybean under drought and Fleurat-Lessard et al. (2005) demonstrated the immunolocalization of PIP1 and PIP2 in soybean root nodules, they did not classify PIP1 and PIP2 into further isoforms. Recent numerous studies have investigated the relationship between water relations and gene expression and/or protein levels of PIP1 or PIP2 subfamilies under various environments in many plants, such as Arabidopsis thaliana (Martre et al., 2002), tobacco (Nicotiana tabacum) (Mahdieh et al., 2008; Mahdieh and Mostajeran, 2009), rice (Oryza sativa) (Sakurai et al., 2005, 2008), common bean (Phaseolus vulgaris) (Arora et al., 2006), maize (Zea mays) (Gorska et al., 2008; Parent et al., 2009), poplar (Populus trichocarpa) (Secchi and Zwieciecki, 2010) and olive (Olea europaea) (Secchi et al., 2007). In contrast, the relationship between AQP gene expression and water relations in soybean has not been reported. Recently, Sadok and Sinclair (2010) indicated the existence of AQP-mediated water transport in the leaves of soybean by using AQP inhibitor (cycloheximide, mercury chloride and silver nitrate). Although their study suggests new avenues of research on water relations in soybean, more detailed studies are needed to understand the unifying association between AQPs and water relations.

In the present study, we focused on the PIP2-type AQPs of soybean, because PIP2s have relatively higher water permeability and plasma membrane is the limiting step in the water transport through the cells. The aims of this study are (i) to find PIP2 isoforms and (ii) to analyze the expression levels of these genes under normoxic and hypoxic conditions as the first step.

Materials and Methods

1. Retrieval and phylogenetic analysis of GmPIP2 genes

Putative AQP genes from the genome sequence of soybean (cv. Williams 82) were found by Phytozome v5.0 (http://www.phytozome.net/) based on the cDNA sequence similarity with GmPIP2;2 (accession number; AP907701.1). A phylogenetic analysis was conducted for their deduced amino acid sequences by using the Clustal W program (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and compared with the PIP2s of a dicotyledonous plant, Arabidopsis thaliana and a legume plant, Medicago truncatula. The results were displayed using the Tree View program (Page, 1996).

2. Plant material and growth conditions

Soybean (cv. Enrei) was used in all studies. The seeds were sown in wet vermiculite and incubated at 28°C for 24 hr to promote germination. Pre-germinated seeds were sown in plastic cups (about 200 mL) filled with soil and grown for 6 d in a growth chamber (14 hr light: 10 hr dark cycle, 25°C, 70% humidity and a photon flux density of about 700 μmol s⁻¹ m⁻²). Seven-day-old seedlings were carefully washed with tap water to remove soil and moved to an aerated hydroponic solution (3.5 L containers; one plant per container). The hydroponic containers were covered with 1.5-cm-thick styrofoam boards and the seedlings were fixed in an upright position using plugs of soft polyurethane. A modified Hoagland solution [0.6 mM KNO₃, 0.4 mM Ca(NO₃)₂, 0.2 mM NH₄H₂PO₄, 0.1 mM MgSO₄; micronutrients: 5.4 μM Fe(III)-EDTA, 5 μM KCl, 2.5 μM H₂BO₃, 0.2 μM MnSO₄, 0.2 μM ZnSO₄, 0.05 μM CuSO₄, and 0.05 μM H₂MoO₄] was used. The pH of the solution was adjusted daily to 5.3 with 1 N NaOH or 1 N HCl and the hydroponic solution was renewed every two days. The plants were grown in the same growth chamber as described above for 5 days to acclimatize to the solution culture. Six days after transplanting (DAT), two treatments were carried out (control and hypoxic). The nutrient solution was continuously aerated in the control to give it a saturated O₂ concentration, and N₂ gas was bubbled at a flow rate of 30 mL min⁻¹ in the hypoxic treatment. Hypoxic treatment was performed 1 hr after the beginning of the light period. The dissolved oxygen concentration in the hydroponic medium was monitored by using a dissolved O₂ meter at each measurement time. The dissolved O₂ concentration in the control was stable throughout the experiment, and was on average 7.61 mg L⁻¹, but it decreased to 0.73 mg L⁻¹ in hypoxic treatment within 3 hr after the start of hypoxic treatment and thereafter the values kept decreasing and finally reached 0.18 mg L⁻¹ (Fig. 1). This result confirmed that hypoxic stress was imposed on plants in the hypoxic treatment throughout the experiment.
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3. RNA extraction and quantitative real-time RT-PCR

The apical half of roots of three replicates was harvested at 3, 6, 12, and 27 hr after the beginning of the hypoxic treatment, immediately frozen in liquid nitrogen, and kept at −80°C. The frozen samples of the roots were ground to a powder in liquid nitrogen with a mortar and pestle. Total RNA was extracted from the frozen powdered tissue with the RNeasy Plant Mini kit (Qiagen Valencia, CA, USA) with DNase treatment according to the manufacturer’s protocol. Total RNA was quantified by spectrophotometry at 260 nm. A portion (800 ng) of the total RNA from each sample was reverse-transcribed to cDNA in a 20 μL reaction volume using an iScript™ cDNA Synthesis Kit (Bio-RAD, Hercules, CA, USA) according to the manufacturer’s protocol. Real-time quantitative PCR was performed using a cDNA product corresponding to 20 ng of total RNA in a 20 μL reaction volume using the iQ™SYBR Green Supermix (Bio-Rad) on a MyiQ™ Single-color real-time PCR detection system (Bio-Rad). The PCR conditions were as follows: 95°C for 3 min, then 40 cycles of 95°C for 30 s and 58°C for 30 s and 72°C for 30 s. The quantity of each transcript was calculated using a standard curve which was produced by using the dilution series of each cDNA as a template. To normalize the gene expression, we used 18S rRNA (accession number: X02623) as an internal control. The primer set for each gene used is listed in Table 1. For the accurate amplification of each specific target gene among the highly homologous PIP2 family, we carefully designed the primer sets for each gene, except GmPIP2;2, based on the sequences corresponding to 3’-untranslational region. Although the primer sets for GmPIP2;2 were designed based on the sequences corresponding to translational region, primers amplified a target gene only (see below). To test the suitability of these primers, the specificity and identity of the PCR products were monitored after each PCR run by melting curve analysis of the reaction products, which can distinguish the gene-specific PCR products from the non-specific PCR products. The temperature of PCR products was elevated from 55 to 95°C at a rate of 0.5°C per 30 s, and the resulting data were analyzed by using the MyiQ software. If only one single peak with a characteristic melting curve was observed for each sample, the RT-PCR reaction produced a product specific to the primers used for the reaction. To further confirm that the primer sets produced only the target genes, we separated the RT-PCR products by electrophoresis and visualized them in a 1% agarose gel.

4. Statistical analysis

Student’s t-test was carried out to separate means between control and hypoxic treatments at each measuring time with SPSS software (SPSS 19.0, SPSS Inc., Chicago, IL).

Results and Discussion

One of the factors reducing soybean yield is hypoxic or anoxic stress caused by heavy rainfall or over-irrigation (Heatherly and Pringle, 1991). In Japan, the optimal sowing time of soybean corresponds to the rainy season (from middle June to middle July). Furthermore, over 80% of soybean is cultivated in the field converted from paddy fields where drainage is poor (Araki, 2006).

Table 1. Gene-specific primer pairs used in this study.

| Gene      | Primer   | Primer sequence |
|-----------|----------|-----------------|
| GmPIP2;2  | Forward  | 5'-ACCAAAATCCCCGGTAACAC-3' |
|           | Reverse  | 5'-ATTCCGGCAGTGCAGTAAAC-3' |
| Glyma12g29510 | Forward | 5'-GTCTCTGTCATCOCACAAATACAG-3' |
| Glyma12g08040 | Forward | 5'-TGCGGAAAGGCGTATGACA-3' |
| Glyma11g20000 | Forward | 5'-GTCGAGTGATGTCGATCTG-3' |
| Glyma11g20600 | Reverse | 5'-ACCTAATTTGTTTGGCCTTATC-3' |
| 18SrRNA    | Forward  | 5'-TGATTAACAGGACACTGTCG-3' |
|           | Reverse  | 5'-ACGGTATCTGATGTCGCTTG-3' |
Therefore, soybean seedlings are often subjected to flooding stress. Short duration (2 to 4 d) of flooding stress at vegetative stage can reduce growth and yield of soybean (Scott et al., 1989). Despite the fact that hypoxic or anoxic stress is firstly imposed on the root systems, few studies have addressed the effects of these stresses on the root physiology (Tournaire-Roux et al., 2003; Araki, 2006; Bramley et al., 2010). Recent studies have revealed that the reduction of root Lp under anoxia stress was caused by the closure of AQP gate (Tournaire-Roux et al., 2003). Except these reports, little information is available for root physiology under low oxygen stress. Furthermore, information about AQP of soybean is still limited, as compared with other plant species, such as rice (Sakurai et al., 2005; 2008), Arabidopsis (Martre et al., 2002) and maize (Ehlert et al., 2009). It is widely reported that, among AQP families, PIP2 type AQPs possess higher water permeability than others in many plant species (e.g., Sakurai et al., 2005). Therefore, we focused on the PIP2 type AQP genes as a first step of root physiology for soybean under low oxygen stress.

By mining the database of soybean PIP2 genes, we identified three putative PIP2 genes (gene name in Phytozome: Glyma12g29510, Glyma12g08040 and Glyma11g20600). The cDNAs of Glyma12g29510, Glyma12g08040 and Glyma11g20600 are 867 bp, 867 bp, 864 bp and 864 bp in length, corresponding to the 287, 287, 286 and 286 amino acids, respectively (Fig. 2). The deduced amino acid sequences of the cDNAs identified from soybean included features typical of MIP proteins. The sequences obtained in this study contained residues conserved in other PIP families. PIP2 type AQPs possess higher water permeability than others in many plant species (e.g., Sakurai et al., 2005). Therefore, we focused on the PIP2 type AQP genes as a first step of root physiology for soybean under low oxygen stress.

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charged) at Loop D was replaced by alanine or asparagine residue (negatively charged) was decreased and not significantly decreased by low cytosolic pH, respectively. From the results, they concluded that anoxia stress decreased the cytosolic pH, the decreased cytosolic pH proteins, the repeated NPA motif and six putative transmembrane domains, as reported by Wallace and Roberts (2004) and Forrest and Bhave (2008). They also contained histidine residues at Loop D, which was shown to be involved in pH gating in other plant AQPs (Luu and Maurel, 2005; Törnroth-Horsefield et al., 2006). At the amino acid level, the sequences of soybean AQP share a high percentage similarity with different AQP proteins. The predicted amino acid sequence of the *GmPIP2;2* gene has the highest similarity with those of *Glyma12g29510* (97.9%) and *MtPIP2;1* (87.3%). For the amino acid sequence of *Glyma12g08040*, the highest similarity is shared with that of *Glyma11g20600* (98.3%). The high homology indicated that the identified putative GmPIPs are isoforms of GmPIP2s. Likewise, the deduced amino acid sequences of *GmPIP2s* and *AtPIP2s* share between 75.6% and 82.9% similarity. Phylogenetic analysis of the soybean PIP2 sequences was performed together with AQP sequences from *Arabidopsis thaliana* and five PIP2s proteins from legume plants (soybean and *Medicago truncatula*) were obviously separated into two groups.

Tournaire-Roux et al. (2003) showed that root Lp, of *Arabidopsis thaliana* was decreased by anoxic stress even at 0.5 hr after anoxia stress onset and the reduction of Lp, coincided well with the decrease of cytosolic pH. They also demonstrated that the water permeability of normal and mutated AtPIP2;2 whose histidine residue (positively charged) at Loop D was replaced by alanine or asparagine residue (negatively charged) was decreased and not significantly decreased by low cytosolic pH, respectively. From the results, they concluded that anoxia stress decreased the cytosolic pH, the decreased cytosolic pH.

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**Fig. 3.** Phylogenetic analysis of the deduced amino acid sequences of soybean aquaporins with those of *A. thaliana* and *M. truncatula*. The scale bar of 0.10 is equal to 10% sequence divergence.

**Fig. 4.** Time course of gene expression of *GmPIP2;2* (a), *Glyma12g29510* (b), *Glyma12g08040* (c) and *Glyma11g20600* (d) in the control (open circle and dashed line) and hypoxic treatments (N2; closed circle and bold line) after the beginning of hypoxic treatment. Data are shown as mean ± S.E. of three biological replicates. *.*, ** and *** indicate significant differences among treatments at 5, 1 and 0.1%, respectively. Black and white bars below the graph indicate dark and light time of chamber, respectively.
caused the protonation of histidine residue at Loop D, the 
protonation of histidine residue at Loop D caused closure 
of the gate of AtPIP2;2 and the closed gate of AtPIP2;2 
resulted in the reduction of root Lp. Fischer and 
Kaldenhoff (2008) investigated the effect of cytosolic pH 
on the water permeability of NiPP2;1 by using mutant 
tobacco NtPIP2;1 whose histidine residues at Loop D was 
replaced by alanine. They reported that water permeability 
of the mutated NtPIP2;1 was not affected by cytosolic pH, 
although that of normal NtPIP2;1 decreased as cytosolic 
ph became acidic. Their results supported the conclusion 
by Tournaire-Roux et al. (2003), because GmPIP2s identified 
here possess the histidine residue at Loop D (Fig. 2).

The time-course expression of GmPIP2;2, Glyma12g29510, 
Glyma12g08040 and Glyma11g20600 in the control and 
hypoxic plants is shown in Fig. 4. The expression patterns 
of all GmPIP2s indicated clear diurnal changes in the 
controls, as observed in other plant species, such as rice 
(e.g., Sakurai et al., 2005). On the other hand, the mRNA 
levels of all GmPIP2s in the hypoxic plants were 
significantly down-regulated from 30 to 50% of the control 
levels even at 3 hr. Significant (P < 0.05) differences in the 
expression levels of all GmPIP2s between the control and 
hypoxic plants were also observed at 6 and 12 hr. The 
mRNA levels of all GmPIP2s of the control plants at 27 hr 
recovered to the same level as those at 3 hr, but this 
phenomenon was not observed in the hypoxic treatment. 
As described above, the root Lp, was decreased by anoxic 
stress as early as 0.5 hr after stress onset (Tournaire-Roux et 
al. 2003). Our results can draw the hypothesis that water 
permeability of roots of soybean is immediately decreased 
by low oxygen stress via transcriptional suppression of 
GmPIP2s. Furthermore, our results suggest co-regulation of 
the four GmPIP2 genes by both diurnal change and oxygen 
concentration around roots systems, though the genes are 
located in distinct loci in soybean genome. If AQPs encoded 
by the genes examined in this study are functional, the 
AQPs may coordinately regulate water transport in soybean 
roots.

In the present study, we identified three GmPIP2 genes 
and expression changes of the genes under hypoxic 
conditions. Whether the identified GmPIP2s encoding 
AQPs possess water permeability remains unknown. The 
biochemical activities of the soybean AQPs under hypoxic 
conditions also remain unclear. Further studies are needed 
to elucidate the detailed root physiology of soybean under 
low oxygen stress.

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