

Over-expression of a Barley Aquaporin Increased the Shoot/Root Ratio and Raised Salt Sensitivity in Transgenic Rice Plants

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Barley HvPIP2;1 is a plasma membrane aquaporin and its expression was down-regulated after salt stress in barley [Katsuhara et al. (2002) *Plant Cell Physiol.* 43: 885]. We produced and analyzed transgenic rice plants over-expressing barley HvPIP2;1 in the present study. Over-expression of HvPIP2;1 increased (1) radial hydraulic conductivity of roots ($L_{p,r}$) to 140%, and (2) the mass ratio of shoot to root up to 150%. In these transgenic rice plants under salt stress of 100 mM NaCl, growth reduction was greater than in non-transgenic plants. A decrease in shoot water content (from 79% to 61%) and reduction of root mass or shoot mass (both less than 40% of non-stressed plants) were observed in transgenic plants under salt stress for 2 weeks. These results indicated that over-expression of HvPIP2;1 makes rice plants sensitive to 100 mM NaCl. The possible involvement of aquaporins in salt tolerance is discussed.

Keywords: Aquaporin — Barley — Salt stress — Transgenic rice — Water transport.

Abbreviations: DW, dry weight; FW, fresh weight; Jr, water flux in root per unit time; $L_{p,r}$, radial hydraulic conductivity of roots; PIPs, plasma membrane intrinsic proteins; RGR, relative growth rate.

The nucleotide sequences reported in this paper have been submitted to DDBJ under accession numbers AB009307 (*HvPIP2;1*, formerly *BPW1*), AB009308 (*HvPIP1;3*, formerly *BPW2*), and AB009309 (*HvPIP1;5*, formerly *BPW3*).

Introduction

Water transport is one of the most basic functions of all plant cells. This function is, however, easily disturbed by salt stress (Kramer 1983). If cellular water uptake and long-distance water transport in plants are seriously disturbed, many important functions such as photosynthesis and the maintenance of turgor will be disrupted or lost. Therefore, plant cells must develop adaptive mechanism(s) of water transport if they are to survive under salt stress.

It is established theoretically and experimentally that the amount of water transport depends on two parameters. One is

the motive force generated by an osmotic gradient (that is, the difference in water potential between two compartments), and the other is water permeability (Kramer 1983). At the cellular level, the former can be restored by a decrease in intracellular water potential with the accumulation of inorganic and/or organic materials (compatible solutes) when extracellular water potential is reduced by salt stress (Greenway and Munns 1980). The over-expression of compatible solutes was demonstrated by many researchers to maintain the turgor for cell growth under salt stress and to maintain water uptake, resulting in an increase in salt tolerance (Tarczynski et al. 1993, Kishor et al. 1995, Hayashi et al. 1997, Nomura et al. 1998)

As for the second parameter, water-permeable paths of plant tissue consist of an apoplastic (or extracellular) path and cell-to-cell path (including symplastic and transcellular paths, Steudle and Peterson 1998). In the cell-to-cell path, which is considered important for regulating plant water transport during adaptation to external changes in the hydraulic environment such as salt stress, recent results are indicating that most of the conductance of water is performed by aquaporins which are membrane proteins forming water channels (Tyerman et al. 1999, Maurel and Chrispeels 2001). In order to understand the cellular mechanism of salt tolerance and water transport, aquaporins under salt stress should be investigated. Such an approach was reported elsewhere (Uno et al. 1998, Suga et al. 2002, Morillon and Lassalles 2002). Some aquaporin genes identified previously were demonstrated to be up-regulated or down-regulated by salt or drought stress according to a review by Tyerman et al. (2002). Consequently, we identified and analyzed three aquaporin genes (*HvPIP1;3*, *HvPIP1;5* and *HvPIP2;1*) in the plasma membrane type subfamily in barley seedlings (Katsuhara et al. 2002). We found that the expression of *HvPIP2;1* was down-regulated by salt stress of 200 mM NaCl. The expression of *HvPIP1;3* and *HvPIP1;5* was constantly lower than that of *HvPIP2;1* and almost un-influenced by salt stress. These results suggested that *HvPIP2;1* is involved in the mechanism of salt tolerance in barley. High water-permeable activity was confirmed in *Xenopus leavis* oocytes expressing HvPIP2;1 (Katsuhara et al. 2002).

In the present study, we introduced the *HvPIP2;1* gene into rice, which belongs to the same family as barley, and successfully produced transgenic plants over-expressing barley

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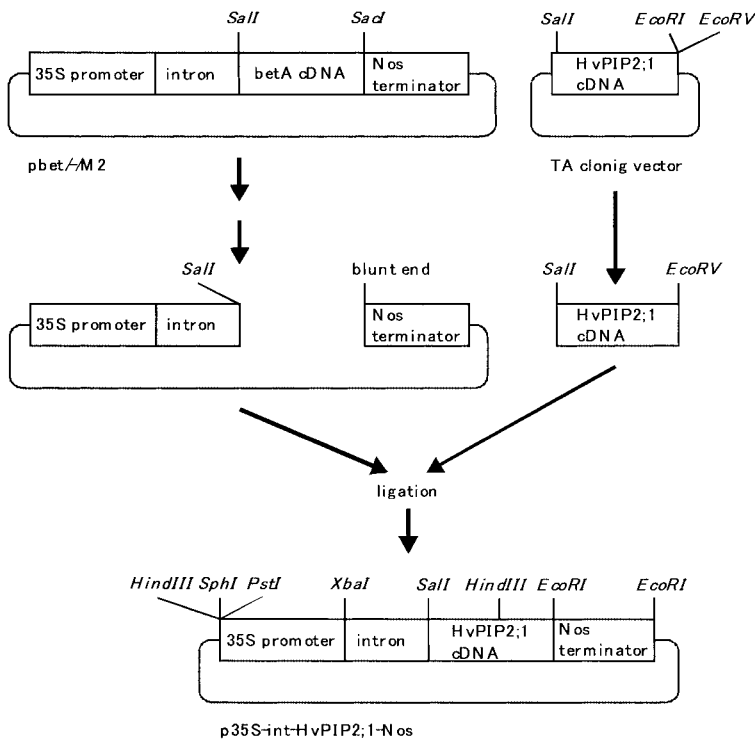


Fig. 1 Construction and restriction enzyme sites of p35S-int-HvPIP2;1-Nos. See “Materials and Methods” for details on the origin of the intron and vector pbet-/M2.

HvPIP2;1. Over-expression of aquaporin is speculated to increase root water permeability which, theoretically, has opposing effects. Excess aquaporins may be advantageous for water uptake under a reduced osmotic gradient between soil and root cells when intracellular water potential is kept below the extracellular water potential, but it may enhance water loss beyond the equilibration of the water potentials (that is, if extracellular water potential drops below the intracellular water potential). Therefore such transgenic plants are expected to be a powerful tool for revealing the role of aquaporins in plant–water relations.

To date, only a few studies have altered the aquaporin gene expression to change cellular membrane water permeability (Kaldenhoff et al. 1998, Barrieu et al. 2000, Siefritz et al. 2002, Aharon et al. 2003). A reduction in aquaporin expression using the antisense technique in *Arabidopsis thaliana* or tobacco resulted in an increase in (1) root mass, (2) root

hydraulic conductivity, and (3) sensitivity to osmotic stress (Kaldenhoff et al. 1998, Siefritz et al. 2002). Recently, Aharon et al. (2003) reported transgenic tobacco plants over-expressing an *A. thaliana* aquaporin, PIP1b. These plants grew better under favorable conditions but not under salt or drought stress. Chaumont et al. (2000) reported that PIPs in the class 2 subfamily showed high water channel activity. HvPIP2;1 is classified into this subfamily. However, most PIPs in the class 1 subfamily showed little or no water channel activity (Chaumont et al. 2000). We also confirmed that there was almost no increase in water permeability of *Xenopus laevis* oocytes expressing barley *HvPIP1 : 3*, a class 1 PIP (unpublished data). Nevertheless, as well as other *A. thaliana* class 1 PIPs, *AtPIP1b*, with which Aharon et al. (2003) produced a transgenic tobacco plant, increased the water permeability of oocytes 5- to 8-fold (Kammerloher et al. 1994).

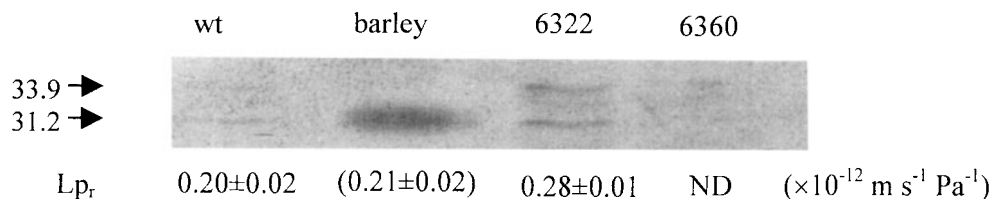


Fig. 2 Expression of *HvPIP2;1* and radial hydraulic conductivities of root (Lp_r). Total root protein (20 μg) was subjected to Western analysis with anti-HvPIP2;1 antibody. Estimated molecular sizes (kD) are indicated at the left. Note that 2 or 3 bands were often recognized with the antibody used in this experiment as previously described (Katsuhara et al. 2002). See “Materials and Methods” for the estimation of Lp_r . Data are means with SD. Barley Lp_r was cited from the data after first infiltration in Table 1 of Tazawa and Okazaki (1997). wt, 6322, and 6360 indicates non-transgenic rice, transgenic line 6322, and transgenic line 6360, respectively. ND, not determined.

Table 1 Plant mass, relative growth rate (RGR), and mass ratio of shoot/root of non-transgenic (wt) and transgenic T₂ rice (line 6322)

	Control			Salt stress		
	FW (g)	RGR (%)	Shoot/root	FW (g)	RGR (%)	Shoot/root
wt	26± 7 (n = 4)	148	2.4 (100%) ^a	20±7 (n = 4)	112	1.8 (100%) ^c
6322	30±14 (n = 6)	137	3.5 (146%) ^b	20±9 (n = 7)	97	2.7 (150%) ^{a, b, c}

Salt stress was induced by adding 100 mM NaCl to the hydroponic solution for 2 weeks. Control plants were grown without salt stress for the same period. Data of plant mass (FW) after treatment for 2 weeks are means with SE. See “Materials and Methods” for the calculation of RGR. Means of the mass ratio of shoot/root followed by different letters are significantly different ($P < 0.05$) according to the *t*-test. Relative values of shoot/root in transgenic 6322 plants are shown in parentheses.

Results

Production of transgenic rice plants over-expressing *HvPIP2;1*

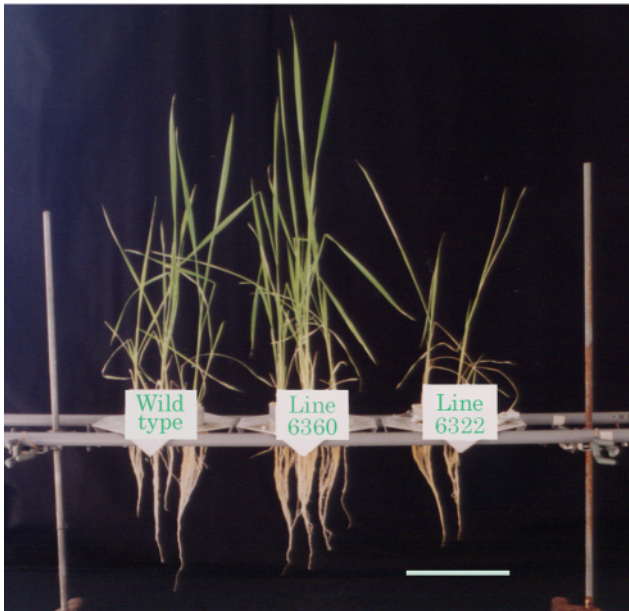
One or more copies of the introduced gene (Fig. 1) were detected with Southern analysis in several independent lines of transgenic T₀ plants (data not shown). In transgenic T₀ cells, a high level of expression of the *HvPIP2;1* transcript and protein was confirmed with Northern and Western analysis (data not shown). Western analysis revealed variation in the level of translational expression in several T₂ lines (Fig. 2). Note that 2 or 3 bands were often recognized with the antibody used in this experiment as previously described (Katsuhara et al. 2002). A line showing a high expression level of *HvPIP2;1* protein (line

6322) was used in subsequent experiments. In roots of this transgenic line, a 40% enhancement of radial hydraulic conductivity (L_p) was observed compared to non-transgenic rice roots. In contrast to line 6322, line 6360 hardly expressed any *HvPIP2;1*.

High mass ratio of shoot/root in transgenic plants

Without salt stress, the shoot height and growth rate, and the mass of transgenic rice plants were almost the same as in non-transgenic plants (Table 1, Fig. 3, 4B). The *HvPIP2;1*-over-expressing line 6322 grew subtly better than non-transgenic plants without stress within 7 d in terms of shoot height (Fig. 4) but not in mass-base relative growth rate (RGR) for 2

(A)



(B)

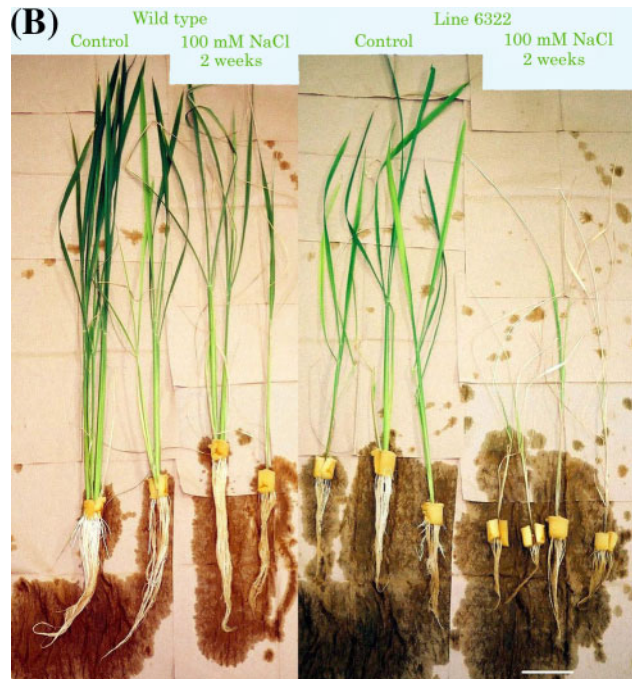


Fig. 3 Appearance of plant after salt stress (100 mM NaCl). (A) Non-transgenic wild type (four individuals), transgenic line 6360 (four individuals), and transgenic line 6322 (two individuals) were cultured hydroponically with 100 mM NaCl for 1 week. (B) Non-transgenic wild type without stress (most left two individuals), with 100 mM NaCl stress (next two individuals), transgenic 6322 line without stress (next three individuals), and with stress (right four individuals) after 2-weeks treatment. The urethane foam around the shoot/root junction was used to support the materials of the hydroponic culture system. Bar = 10 cm.

Table 2 Water content (%) of shoot and root of non-transgenic (wt) and transgenic T₂ rice (line 6322)

	Root		Shoot	
	Control	Salt stress	Control	Salt stress
wt	90.7±0.4 ^{a,b}	92.0±1.0 ^b	81.7±0.3 ^c	76.6±1.1 ^d
6322	89.9±0.4 ^a	89.5±2.8 ^{a,b}	79.0±1.2 ^d	61.2±8.5 ^e

Salt stress was induced by adding 100 mM NaCl to the hydroponic solution. Data are means with SD. Means followed by different letters are significantly different ($P < 0.05$) according to the *t*-test.

weeks (Table 1). However, the mass ratio of shoot/root was significantly higher in the transgenic 6322 plants than non-transgenic plants (Table 1). Dry weight (DW)-base mass ratios of shoot/root were also significantly different between line 6322 and non-transgenic rice (data not shown).

Characteristics of transgenic plants under salt stress

Salt stress (100 mM NaCl) reduced the water content of shoot but root (Table 2). This is why the mass ratio of shoot/root decreased from 2.4 to 1.8 in non-transgenic plants and from 3.5 to 2.7 in the transgenic 6322 line (Table 1).

The change in the amount of HvPIP2;1 during salt stress was analyzed with Western blotting. The relative amount of HvPIP2;1 relative to that of cytochrome *c*, which was used as a constantly expressed internal control, was 0.941 with salt stress (100 mM NaCl) for a week and 0.944 without. Although the expression of HvPIP2;1 was constant with or without salt stress, the growth of the transgenic plants was greatly inhibited when 100 mM NaCl was present in the hydroponic solution. Under salt stress of 100 mM NaCl, non-transgenic plants showed reduced growth but did not die (Fig. 3). Under the same conditions (100 mM NaCl), the transgenic plants stopped growing (Fig. 4), withered and died (Fig. 3). The same results

were obtained when the experiments were repeated. Growth reduction of the transgenic line 6360, in which HvPIP2;1 was hardly detected, was less than in line 6322, and almost the same as in the wild type after exposure to 100 mM NaCl (Fig. 4). When strong salt stress (200 mM NaCl) was applied, no rice plants, transgenic or non-transgenic, could survive (data not shown).

Discussion

High expression levels of HvPIP2;1 protein were confirmed in the transgenic line 6322. Enhanced L_p was observed in roots of these transgenic plants compared to non-transgenic rice. In the transgenic line 6322, HvPIP2;1 in addition to inherent rice aquaporins was considered to be responsible for the increase in L_p.

Without salt stress, the growth of transgenic rice plants was similar to that of non-transgenic plants in the present study. In line 6322, the mass ratio of shoot/root was higher than that in non-transgenic plants. This means that a smaller amount of root can sustain the shoot of a plant over-expressing aquaporin. The same phenotype of a higher mass ratio of shoot/root was observed in transgenic tobacco plants over-expressing aquaporin (Aharon et al. 2003). In contrast, when the plasma membrane-type aquaporins were reduced in the study of antisense *A. thaliana*, the mass ratio of shoot/root was demonstrated to be low, that is, a large root mass was observed in the antisense *A. thaliana* (Kaldenhoff et al. 1998). The observed value for root mass probably reflects the total root surface area. The increase or decrease in aquaporins in transgenic plants is speculated to increase or decrease root water permeability per unit surface areas, resulting in a small or large total root surface area to maintain a constant water uptake by roots, respectively. In the case of transgenic tobacco plants over-expressing PIP1b (Aharon et al. 2003), however, enhanced L_p could be

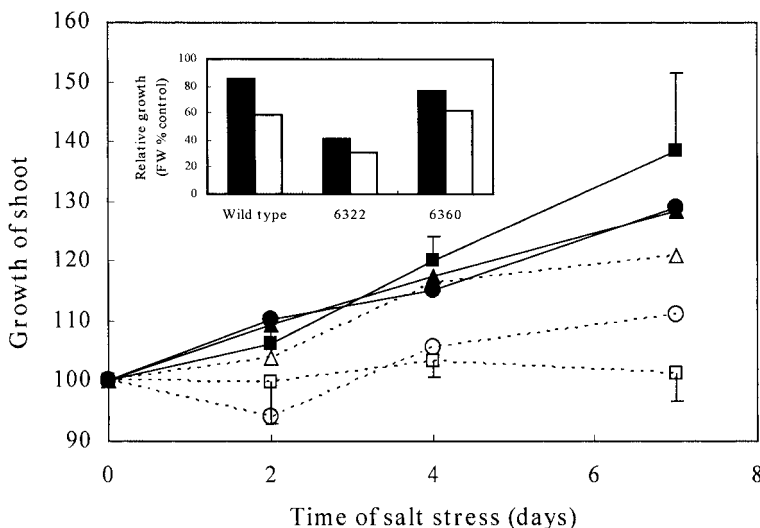


Fig. 4 Effect of salt stress (100 mM NaCl) on shoot height of non-transgenic rice (circles), transgenic line 6322 (squares), and transgenic line 6360 (triangles). The hydroponic solution was supplemented with 100 mM NaCl (open symbols) or not (closed symbols) at day 0. Shoot height at day 0 was normalized to 100. Data are means with SD. To avoid complicating the figure, not all SD values (error bars) are given. Observed shoot heights at day 0 (cm) were 29.2±6.8 for non-transgenic plants without salt stress, 30.2±6.6 for non-transgenic plants with salt stress, 34.8±1.7 for transgenic line 6322 without salt stress, 32.9±2.4 for transgenic line 6322 with salt stress, 36.1±2.1 for transgenic line 6360 without salt stress, and 36.9±3.3 for transgenic line 6360 with salt stress. Inset: reduction of roots (solid) or shoots (open) FW after salt stress treatment (100 mM NaCl) for 2 weeks. Roots or shoot FWs without salt stress were established as 100%. Data were calculated from four, seven and eight samples of non-transgenic, transgenic line 6322, and transgenic line 6360 plants, respectively.

accompanied by an increase in the supply of water from roots to shoots, which was probably the reason why transgenic tobacco plants showed good growth under favorable conditions.

Although lines 6360 and 6322 in the present study were descendants of the same T_0 rice plant, less HvPIP2;1 protein was detected in line 6360 (Fig. 2) because of silencing. Growth reduction in the transgenic line 6360 was almost the same as that in the wild type after salt stress of 100 mM NaCl in contrast to line 6322 which showed a salt-sensitive phenotype. This result indicates that the observed sensitivity to salt depends on the expression of the introduced gene.

In barley seedlings, the expression of HvPIP2;1 (transcript and protein) was suppressed under salt stress (Katsuhara et al. 2002). A decrease in the number of aquaporin molecules after salt stress can effectively prevent water loss from root tissue. In addition to transcriptional regulation, post-translational regulation of aquaporins is also involved in controlling water transport under salt stress (Johansson et al. 2000). In spinach, PM28A, a plasma membrane aquaporin, was made to close through dephosphorylation to prevent water loss under conditions of low external water potential (Kjellbom et al. 1999).

In the transgenic line 6322, *HvPIP2;1* expression was constitutively driven by the 35S-promoter and not down-regulated after salt stress. Loss of the down-regulation of the introduced aquaporin seemed to direct line 6322 to loss water after salt stress. How did loss of water in the shoot occur in the transgenic line? One possible explanation is that the increase in water conductance of leaf cells simply accelerates water loss from transgenic leaves. Another possible explanation is that the highly constant root water permeability caused by the over-expressed aquaporin resulted in a loss of water in the tissue and a lethal disturbance in the supply of water from root to shoot. This may lead to a water shortage in the shoot. Since symplastic water is considered to flow mainly through aquaporins whereas apoplastic water is not (Tazawa and Okazaki 1997, Tyerman et al. 1999), an excess of aquaporin seems to change the symplastic water flow. The supply of water to the shoot is thought to greatly depend on the root symplastic water flow especially around the root endodermis (Kramer 1983, Tyerman et al. 1999). Therefore it is possible that the regulation of root water conductance was lost in transgenic plants under salt stress resulting in a disturbance of the water supply from root to shoot. Because a hydroponic culture system was used in the present study, equilibration between the root apoplastic water and surrounding hydroponic solution could be the reason for the lack of significant change in root water contents after salt stress (Table 2).

Considering both the present results and previous reports, the regulation of aquaporin expression appears to be important for adequate tissue and/or cellular water transport under salt stress. Although the continuous suppression of aquaporin(s) may be disadvantageous to growth under normal conditions, the conditional regulation of some aquaporins seems to be essential for improving salt tolerance in plants.

Materials and Methods

Plant materials, growth conditions, and growth characterization

A japonica rice variety (*Oryza sativa* cv. Kinuhikari) was used in this study. Transgenic T_0 plants (less than 10–14 cm in shoot height) were grown in plant culture bottles as previously described (Shimamoto et al. 1989) and then transplanted to Wagner pots (1/5,000 are) filled with soil (1 : 1 mixture of paddy soil and humus) fertilized with 400 mg/pot each of N, P_2O_5 , and K_2O . Under a controlled environment (25°C, 10 h dark/14 h light with fluorescent lamps of 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$), T_0 and T_1 plants were grown to maturity and seeds were obtained by self-pollination. In all experiments, T_2 plants were analyzed.

Before salt stress treatment, T_2 plants (2 weeks old) were transplanted from soil to a hydroponic culture with aeration in 3.5-liter pots filled with a hydroponic solution (4 mM KNO_3 , 1 mM $NH_4H_2PO_4$, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mg liter⁻¹ sodium-silicate, 1 mg liter⁻¹ Fe as Fe-citrate, 0.5 mg liter⁻¹ B as H_3BO_3 , 0.5 mg liter⁻¹ Mn as $MnCl_2$, 0.05 mg liter⁻¹ Zn as $ZnSO_4$, 0.02 mg liter⁻¹ Cu as $CuSO_4$, and 0.01 mg liter⁻¹ Mo as Na_2MoO_4 , pH 5.5–6.0 with NaOH) under a controlled environment as described above. The hydroponic solution was replaced every week for 4 weeks, and then whole plant fresh weight (FW) was measured before starting salt stress treatment, which was performed by adding 20.5 g of NaCl to 3.5 liter of hydroponic solution (final concentration: 100 mM NaCl). Salt stress treatment was applied for a week and a small amount of root was sampled for isolating protein. After the replacement of the hydroponic solution, plants were grown for another week under the same conditions of salt stress (a total of 2 weeks of salt stress), then whole plant FW was measured again. Relative growth rate (RGR, %) was defined as $100 \times (\text{FW after treatment}) / (\text{FW before treatment})$. To calculate the mass ratio of shoot/root, shoots and roots were harvested separately. After the measurement of FW, they were dried at 100°C for 4 or 5 d until the measurement of DW. Water content (%) was defined as $100 \times (\text{FW} - \text{DW}) / \text{FW}$.

Vector construction and transformation

The entire coding region of *HvPIP2;1* was obtained by digestion of a TA cloning vector (Invitrogen, CA, U.S.A.) containing a full-length cDNA of *HvPIP2;1* (Katsuhara et al. 2002) and was substituted for betaA cDNA in the vector pbet-/M2 (Hayashi and Hayakawa 1998) resulting in the expression vector p35S-int-HvPIP2;1-Nos (Fig. 1). The cauliflower mosaic virus 35S-promoter was used for a constant and strong expression of the transgene. Introduction of the first intron of the castor bean catalase gene was demonstrated to enhance foreign gene expression in rice (Tanaka et al. 1990). Transformation of rice protoplasts with electroporation methods and the selection of a transgenic callus with hygromycin B (Sigma) were performed as previously described (Shimamoto et al. 1989).

Southern, Northern and Western analysis

A fragment of *HvPIP2;1* (0.9 Kb), which covers most of the coding region of the gene, was used as a cDNA probe for Northern and Southern analyses which were performed as previously described (Hayakawa et al. 1992). The generation of anti-HvPIP2;1 antibody, isolation of total protein and Western analysis were performed as previously described (Katsuhara et al. 2002).

Estimation of radial hydraulic conductivities of root

A double-chamber volumeter was used to measure L_p after some modification of the method of Katsuhara et al. (2003). Primary roots 4 cm in length were cut and infiltrated with control solution (0.1 mM each of NaCl, KCl, and $CaCl_2$) in a syringe with small static pressure to remove apoplastic gas that reduces the effective surface for water permeability. Then part of the individual root (2 cm in length)

was set in one chamber and separated from the other part hydraulically with a mixture of Vaseline and lanolin. Initially, a control solution was added to both chambers. Transroot osmosis was induced by replacing the control solution in one chamber with control solution supplemented with 200 mM sorbitol (the osmotic potential was calculated to be 0.49 MPa). The volume of water flow was calculated from the movement of air bubbles in the capillary connected to the other chamber. L_p was calculated as

$$L_p = J_v / [S \times OPD]$$

based on the volume of water flow per unit time (J_v), surface area of the root in one chamber (S), and the osmotic pressure difference between two chambers (OPD). In the present study, OPD was 0.49 MPa. S was calculated from the root length (2 cm) and root diameter which was measured under the microscope with a proof ocular micrometer.

Acknowledgments

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