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Aquaporin Isoforms Responsive to Salt and Water Stresses and Phytohormones in Radish Seedlings

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Aquaporins in the plasma and vacuolar membranes play a key role in the intercellular and intracellular water transport in plants. First, we quantitated the absolute amounts for mRNAs of eight aquaporin isoforms in hypocotyls of radish seedlings. Then, we investigated the effects of salt and water stresses (150 mM NaCl, 300 mM mannitol and 20% polyethylene glycol) and phytohormones (gibberellic acid, abscisic acid and brassinolide) on the mRNA and protein levels of aquaporins in the plasma membrane (RsPIP1-1, 1–2, 1–3, 2–1, 2–2 and 2–3) and vacuolar membrane (RsTIP1-1 and 2–1). The mRNA and protein levels of RsTIP1-1, RsTIP2-1, RsPIP1-1, RsPIP1-2 and RsPIP1-3 were comparatively constant. In contrast, mannitol treatment altered the mRNA levels of *RsPIP2-1*, *RsPIP2-2* and *RsPIP2-3* in roots. Immunoblot analysis showed that the RsPIP2-1 protein level was increased by NaCl treatment and decreased by treatment with mannitol and polyethylene glycol. Gibberellic acid and abscisic acid suppressed the levels of mRNAs of *RsPIP2-1*, *RsPIP2-2* and *RsPIP2-3* and the protein level of RsPIP2-1 in roots. On the other hand, the protein levels of RsPIP1-group members and RsTIPs were scarcely changed by these phytohormones. In the case of hypocotyls and cotyledons, the mRNA and protein levels of eight isoforms were not markedly affected by any treatment. These results indicate that aquaporins in the root, especially the RsPIP2 group, may be a stress responsive type of aquaporin at least in the protein level.

Keywords: Aquaporin – Phytohormone — Plasma membrane — Salt stress — Water channel.

Abbreviations: ABA, abscisic acid; BL, brassinolide; GA_3, gibberellic acid; PEG, polyethylene glycol; RsPIP, radish plasma membrane aquaporin; RsTIP, radish vacuolar membrane aquaporin.

Introduction

Aquaporins comprise a large protein superfamily and have been found in various organisms including bacteria, yeasts and mammals (Borgnia et al. 1999, Johanson et al. 2001, Maurel 1997, Tyerman et al. 2002). Recently, the electron and X-ray crystallographic studies of mammalian aquaporins have revealed the structural basis of substrate recognition and rapid water transport (de Groot and Grubmuller 2001, Sui et al. 2001, Zhu et al. 2001). In plants, aquaporins are thought to play a key role in the intercellular and intracellular water transport. Plant aquaporins are distinct from those of mammals in three respects: (1) they are localized in two separate membranes; (2) they have many isoforms; and (3) they exist in large quantities in the membranes (Johanson et al. 2001, Maeshima 2001, Maurel 1997, Tyerman et al. 2002).

Arabidopsis thaliana contains 35 genes for aquaporins (Chrispeels et al. 1999, Johanson et al. 2001) and maize 31 genes (Chaumont et al. 2001). Several isoforms may be expressed in tissue- and time-specific manners and the others are constitutive. Some isoforms are expected to respond to internal and external stimuli. Many groups have investigated the effects of salt and water stresses on the aquaporin genes to elucidate how aquaporins regulate the water flow, water reservation and adaptation to water stresses in plants. Expression of aquaporin genes has been reported to be induced by salt and water stresses (Fray et al. 1994, Guerrero et al. 1990, Liu et al. 1994, Mariaux et al. 1998, Uno et al. 1998, Yamada et al. 1997). On the other hand, aquaporins are down regulated both transiently and permanently in several plants (Yamada et al. 1995, Mariaux et al. 1998, Li et al. 2000). Although the transcriptional regulation has been extensively investigated, information on the protein accumulation under stress conditions is limited.

We have been conducting a series of experiments to quantify mRNAs and proteins of plasma membrane intrinsic proteins (PIPs) and tonoplast intrinsic proteins (TIPs) in radish organs (Suga et al. 2001). From the present report, we will use the names of radish aquaporin isoforms proposed by Johanson et al. (2001); RsPIP1-1 (previous name, PAQ1), RsPIP1-2 (PAQ1b), RsPIP1-3 (PAQ1c), RsPIP2-1 (PAQ2), RsPIP2-2 (PAQ2b), RsPIP2-3 (PAQ2c), RsTIP1-1 (γ-VM23), RsTIP2-1 (δ-VM23). Among the six isoforms of RsPIPs and two isoforms of RsTIPs, the levels of mRNA of *RsPIP2-1*, *RsPIP2-2* and *RsPIP2-3* and of RsPIP2-1 protein varied with the organ and the stage of growth (Suga et al. 2001). The protein levels of the RsPIP1 group and RsTIPs on a membrane protein basis were retained at a relatively constant level. Therefore, we estimate that the RsPIP2 group may be responsive to physiological stresses. In this study, we investigated the gene expression and protein accumulation of aquaporins under stress condi-

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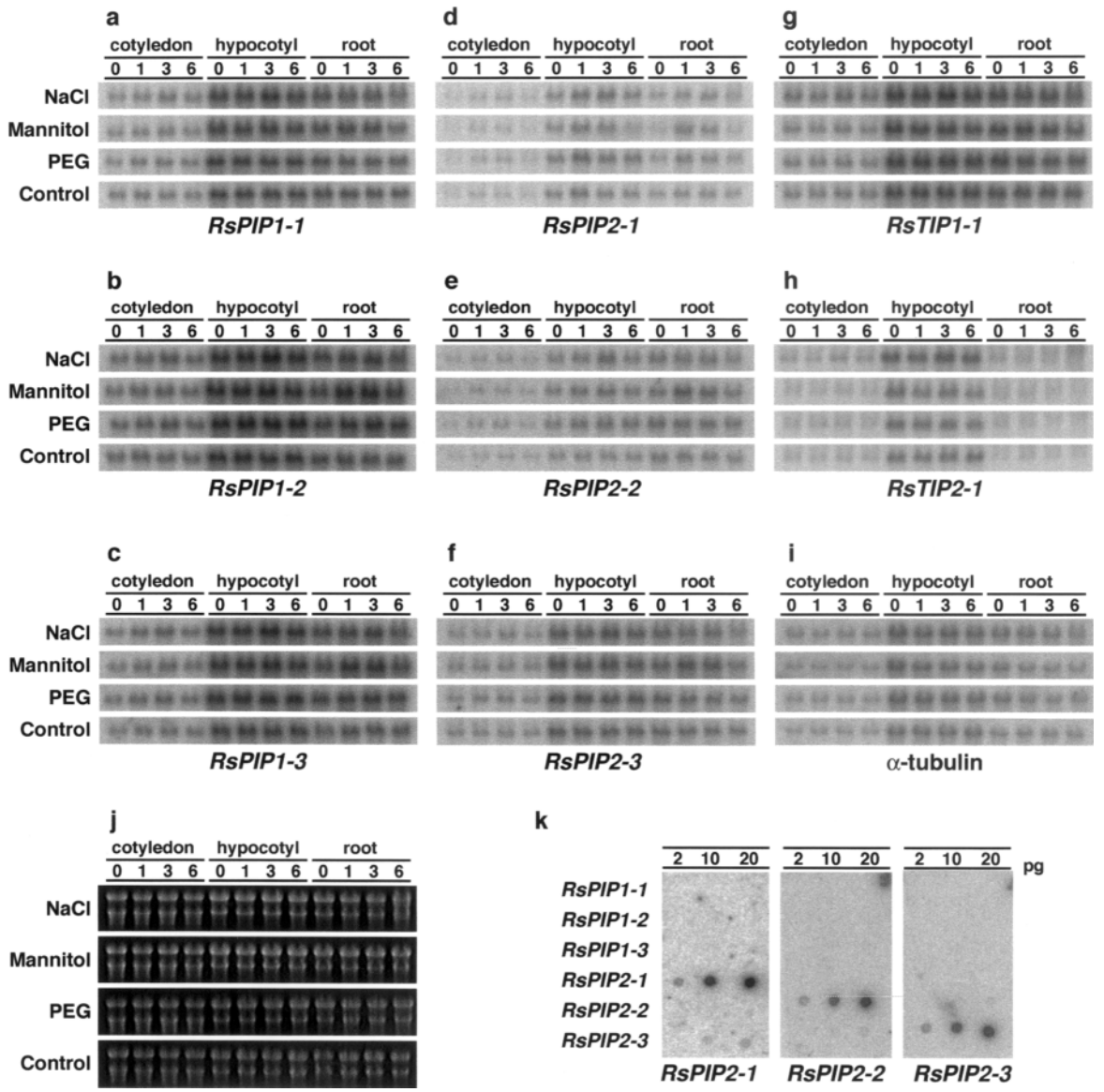


Fig. 1 Relative mRNA levels of *RsPIP1-1* (a), *RsPIP1-2* (b), *RsPIP1-3* (c), *RsPIP2-1* (d), *RsPIP2-2* (e), *RsPIP2-3* (f), *RsTIP1-1* (g), and *RsTIP2-1* (h) under various stress conditions. Five-day-old seedlings were treated with 150 mM NaCl, 300 mM mannitol and 20% PEG for the indicated time (h). Aliquots (10 μg) of total RNA extracted from cotyledons, hypocotyls and roots were subjected to Northern blot analysis. α-Tubulin mRNA was examined as a control mRNA (i). The agarose gel was stained with ethidium bromide to detect rRNAs (j). (k) Specificity of the DNA probes. The indicated amounts of plasmid DNAs containing aquaporin cDNAs were spotted onto a nylon membrane and hybridized with labeled DNA probes, *RsPIP2-1* (left), *RsPIP2-2* (center) and *RsPIP2-3* (right).

tions to test this hypothesis. We also examined the effect of phytohormones on the mRNA and protein levels of aquaporins. We found that certain members of the RsPIP2 group responded to salt and water stresses and phytohormones, while the others did not. Furthermore, we quantified the absolute amount of mRNA of each isoform in the hypocotyl. The present paper describes the physiological roles of the aquaporin isoforms with special regard to their individuality.

Results

Changes in mRNA levels of aquaporins under salt and water stresses

In this study, we investigated six isoforms of the plasma membrane aquaporins (RsPIP1-1, 1-2, 1-3, 2-1, 2-2 and 2-3) (Suga et al. 2001) and two isoforms of the vacuolar membrane aquaporins (RsTIP1-1 and 2-1) (Higuchi et al. 1998) of radish. We examined the effects of salt and osmotic stresses on their mRNA levels in seedlings treated with 150 mM NaCl, 300 mM

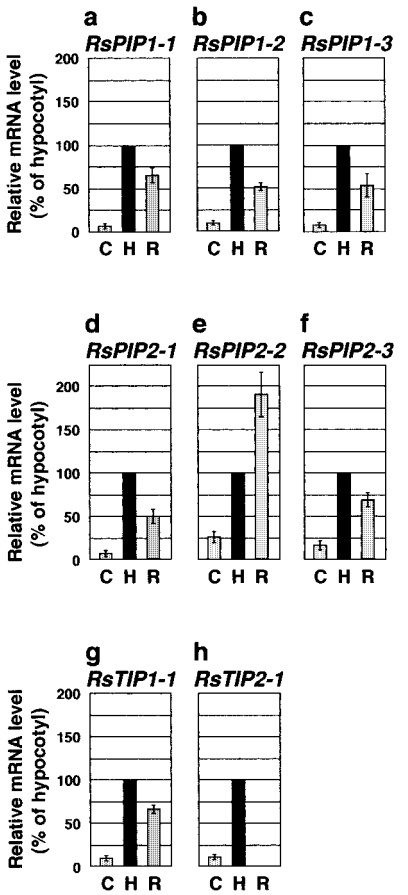


Fig. 2 Relative accumulation of aquaporin mRNAs in cotyledons (C), hypocotyls (H) and roots (R) of radish seedlings. Total RNA was extracted from different organs of 5-day-old seedlings grown under normal conditions and aliquots (10 μg) were subjected to Northern hybridization. The mRNA level is expressed as the signal strength on Northern blot relative to that of the hypocotyl. (a) *RsPIP1-1*, (b) *RsPIP1-2*, (c) *RsPIP1-3*, (d) *RsPIP2-1*, (e) *RsPIP2-2*, (f) *RsPIP2-3*, (g) *RsTIP1-1*, (h) *RSTIP2-1*. Values are expressed as mean ± SD calculated for four independent experiments.

mannitol or 20% PEG. Plants absorb neither mannitol nor PEG.

As shown in Fig. 1, the level of mRNA was relatively constant even after treatment with NaCl, mannitol and PEG, although the level varied with the tissue. Among the eight isoforms, the mRNA levels of *RsPIP2-1*, *RsPIP2-2* and *RsPIP2-3* in roots were increased 1.5- to 4-fold of the original level within 1 h after treatment with mannitol, and then decreased thereafter (Fig. 1d–f). In contrast, the treatment with NaCl and PEG did not show such an effect on the mRNA levels of *RsPIP2* members. Mannitol treatment had a similar effect on the transcript levels of *RsPIP1-1*, *RsPIP1-2* and *RsPIP1-3* (Fig. 1a–c). In hypocotyls, the mRNAs of *RsPIP1* and *RsPIP2* groups were slightly increased by the treatment with NaCl. In cotyledons, the mRNA level was relatively low and did not change for at least 6 h. Treatment with these compounds for more than 12 h caused wilting of seedlings. Thus, the effects were investigated at 6 h after treatment.

Here we note the specificity of DNA probes used for the Northern hybridization. In the present study we newly prepared four probes of *RsPIP1-2*, *1-3*, *2-2* and *2-3*, which contained the 3′-untranslated region of cDNA, since the probes prepared previously for these sequences cross-reacted with the related sequence (Suga et al. 2001). The other probes of *RsPIP1-1*, *RsPIP2-1*, *RsTIP1-1* and *RsTIP2-1*, which contained the 5′-untranslated region of cDNA, specifically reacted with the corresponding DNA (Higuchi et al. 1998, Suga et al. 2001). The probes of *RsPIP2-1*, *RsPIP2-2* and *RsPIP2-3* recognized specifically the corresponding sequence (Fig. 1k). The probes of *RsPIP1-2* and *RsPIP1-3* cross-reacted weakly with the DNA of *RsPIP1-3* (signal intensity, 17%) and *RsPIP1-2* (11%), respectively, when the corresponding DNAs were tested by Northern blot hybridization. This is due to the sequence similarity between *RsPIP1-2* and *RsPIP1-3*.

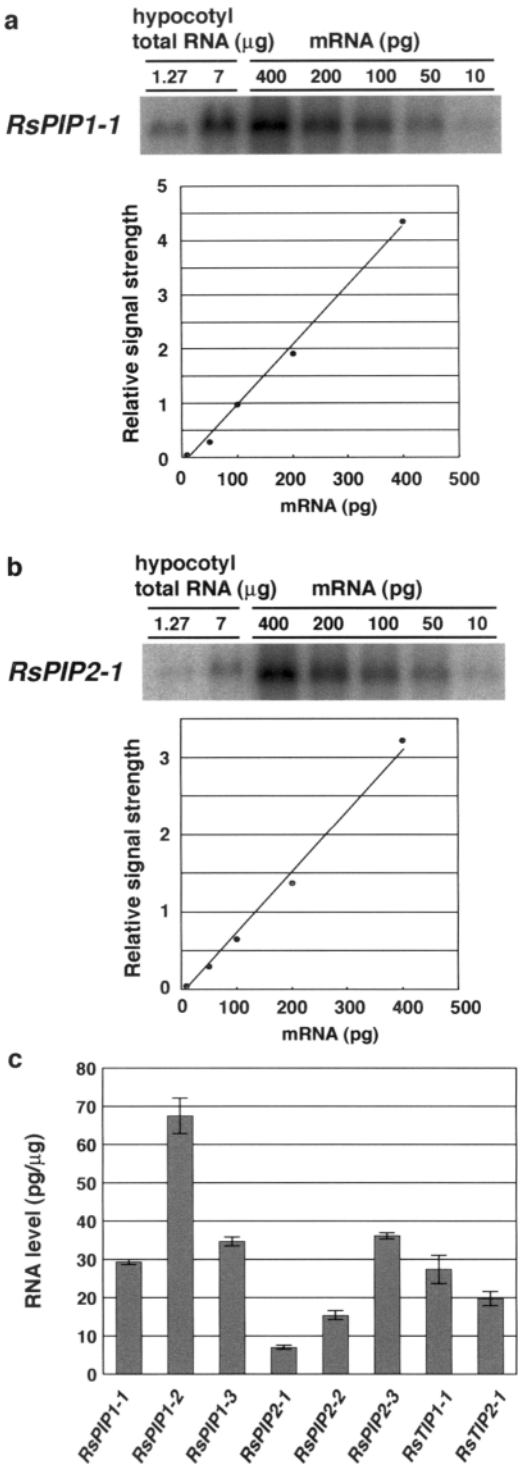
Absolute amount of mRNA of aquaporin isoforms in hypocotyls

The transcript level of most isoforms was high in the hypocotyls and roots and low in the cotyledons of 5-day-old seedlings grown under normal conditions (Fig. 2). In general, Northern blot analysis shows the relative amount of a specific mRNA on the basis of total RNA applied. In this study we determined the absolute amounts of mRNAs of aquaporin isoforms in seedlings by the quantitative Northern blot analysis. Hypocotyls were used as a typical organ because of the presence of mRNAs of all eight isoforms. Sense RNA was prepared from each cDNA of aquaporins and was used as the standard mRNA.

Fig. 3 shows the hybridization images and calibration curves of *RsPIP1-1* and *RsPIP2-1* (panels a and b). The hybridization signal intensity was proportional to the applied amount of standard mRNA up to 400 pg for all isoforms. The mRNA amounts of eight isoforms are summarized (Fig. 3c). The level of *RsPIP1-2* mRNA was the highest (70 pg μg^{-1} of total RNA) and that of the *RsPIP2-1* mRNA was the lowest one (7 pg μg^{-1} of total RNA) in hypocotyls.

Accumulation of aquaporin proteins under stress conditions

For quantification of radish aquaporins, the peptide-specific antibodies to RsPIP1-1 and RsPIP2-1 were prepared as polyclonal antibodies. We obtained the peptide specific antibody to the N-terminal part of RsPIP2-1, which is unique to this isoform (Suga et al. 2001). However, the amino acid sequences are highly conserved among the sequences of three members of the RsPIP1 group with identities of more than 96% (Fig. 4). Even in the N-terminal parts, the RsPIP1-1, RsPIP1-2 and RsPIP1-3 proteins have the same sequence (Suga et al. 2001). Thus, It is impossible in theory to prepare the antibody that recognizes only a single member of the RsPIP1 group. The anti-RsPIP1-1 antibody equally reacts with RsPIP1-1, RsPIP1-2 and RsPIP1-3. In the case of the vacuolar membrane aquaporins, we used the polyclonal antibody to RsTIP pre-



pared previously (Maeshima 1992). The anti-RsTIP antibody mainly reacts with RsTIP1-1 rather than RsTIP2-1 (Higuchi et al. 1998).

We quantified the relative amount of the aquaporin proteins in seedlings grown under various stress conditions. Fig. 5 shows immunoblots of aquaporins in the crude membrane fractions with antibodies to RsPIP1, RsPIP2-1 and RsTIP. The immunostained intensity of RsPIP1s at 30 kDa was equal among the three different tissues (Fig. 5a). The RsPIP2-1 protein was not detected in cotyledons, in which *RsPIP2-1* mRNA was extremely low. RsTIP was detected as a band at 23 kDa in three organs and was abundant in hypocotyls and roots. Treatment with NaCl, mannitol and PEG did not cause a marked change in the protein level of RsPIP1s and RsTIP. However, the NaCl treatment increased the amount of RsPIP2-1 3-fold over the zero-time level (Fig. 5b). The protein level of RsPIP1s in the roots also increased to 150% by NaCl. Under normal conditions, the RsPIP2-1 level was decreased to 73% of the zero-time level. Treatment with either mannitol or PEG enhanced this decrease of the RsPIP2-1 protein (63% on time 6 h).

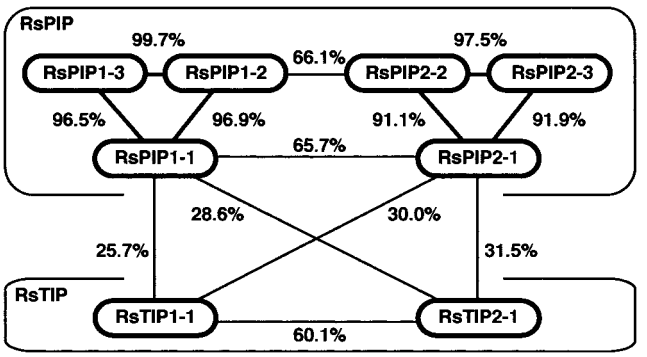


Fig. 4 Amino acid sequence identities among radish aquaporins. The identity is shown above each line. The accession numbers of the DDBJ/EMBL/GenBank database: AB012044 (*RsPIP1-1*), AB030695 (*RsPIP1-2*), AB030696 (*RsPIP1-3*), AB012045 (*RsPIP2-1*), AB030397 (*RsPIP2-2*), AB030698 (*RsPIP2-3*), D84669 (*RsTIP1-1*), AB010416 (*RsTIP2-1*).

Down regulation of mRNA and protein levels of aquaporins by phytohormones

We examined the effects of three types of phytohormones, abscisic acid (ABA), gibberellic acid (GA_3) and brassinolide (BL), on the transcriptional level of aquaporins in seedlings (Fig. 6). None of them markedly changed the mRNA level of any of the aquaporins in either the cotyledons or hypocotyls. However, the mRNA levels of *RsPIP2* group members and *RsTIP1-1* were decreased to less than 30% of the zero-

Fig. 3 Quantification of aquaporin mRNAs in the seedling hypocotyl. Total RNA was extracted from 4-day-old hypocotyls. Standard mRNA was synthesized for each clone. Sample RNA and standard mRNA were applied to the same membrane sheet for the Northern blot hybridization and then the signal strength was determined with an image analyzer. The Northern blots and calibration curves for *RsPIP1-1* (a) and *RsPIP2-1* (b) are shown. Amounts of aquaporin mRNAs on the basis of total RNA are shown by a graph (c). In the case of *RsPIP1-2* and *RsPIP1-3*, the extent of cross-hybridization of the probe to *RsPIP1-3* or *RsPIP1-2* was considered to calculate the absolute amounts. The obtained values are shown as mean ± SD for two independent experiments.

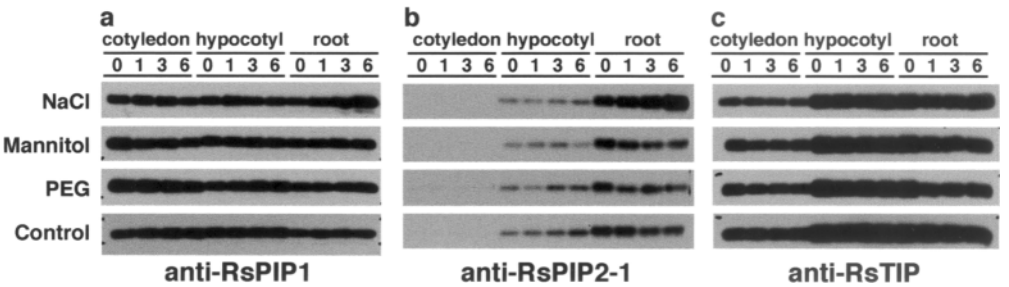


Fig. 5 Effects of salt and osmotic stresses on the accumulation of aquaporin proteins. Five-day-old seedlings were treated with NaCl, mannitol and PEG for the indicated period (h). Aliquots (5 μg) of the crude membrane fractions prepared from cotyledons, hypocotyls and roots were subjected to immunoblotting with antibodies to RsPIP1 (a), RsPIP2-1 (b) and RsTIP (c).

time level in seedling roots treated with ABA, although the levels were constitutive under normal conditions. Treatment with GA_3 at 1 mM (Fig. 6) or 0.1 mM (data not shown) also lowered these mRNA levels.

Fig. 7 shows immunoblots of the crude membrane fractions prepared from seedlings treated with phytohormones. The treatment with GA_3 and ABA lowered the amount of RsPIP2-1 protein to 15% and 45% of the zero-time level, respectively (Fig. 7b). The treatment with BL sustained the original level of RsPIP2-1 protein. Three phytohormones scarcely changed the amount of RsPIP1s and RsTIPs in the three organs with an exception of RsPIP1s in roots treated with GA_3.

Discussion

Aquaporin isoforms investigated in the present study

Radish belongs to the same family of Brassicaceae as *Arabidopsis*. Therefore, the number of aquaporin genes in radish is estimated to be more than 30. Thirty-five aquaporins in *A. thaliana* have been divided into four subfamilies, PIPs (13 members), TIPs (10 members), NIPs (NOD26-like intrinsic proteins, nine members), and SIPs (small basic intrinsic proteins, three members) (Johanson et al. 2001). The PIPs were divided into PIP1 and PIP2 groups, and the TIPs into TIP1 (γ type), TIP2 (δ type) and TIP3 (α type) groups. TIP3s are mainly accumulated in seeds. Soybean NOD26, the NIP prototype, is located in the peribacteroid membrane in root nodules. The physiological roles and intracellular location of the *Arabidopsis* NIPs and SIPs are unknown. Thus, it is reasonable to think that eight isoforms (RsPIP1s, RsPIP2s, RsTIP1 and RsTIP2) investigated in this study represent the major aquaporins in the plasma and vacuolar membranes in the vegetative tissues of radish.

The present study examined the absolute mRNA content of each aquaporin isoform. In seedling hypocotyls, the level of *RsPIP1-2*, which corresponds to *A. thaliana PIP1b* (*PIP1-2*), was the highest among the eight isoforms. This finding is consistent with the report that the *PIP1b* (*PIP1-2*) transcript is the most prominent PIP messenger in *A. thaliana* (Grote et al. 1998). The other isoforms could be divided into the intermediate-level group (*RsPIP1-1*, *RsPIP1-3*, *RsPIP2-3* and *RsTIP1-1*) and low-level group (*RsPIP2-1*, *RsPIP2-2* and *RsTIP2-1*). The mRNA contents varied from 7.0 pg (*RsPIP2-1*) to 70 pg (*RsPIP1-2*) per μg of total RNA. Using the fact that mRNAs constitute about 3.0% (w/w) of the total RNA in the cells, the *RsPIP1-2* mRNA content per total mRNA was calculated to be about 0.27%. The value is not extremely high. Thus we propose that the coordinate and simultaneous expression of several aquaporin genes in the same cell is required to accumulate a large quantity of aquaporin proteins. As a result, the abundance of aquaporins in radish may be maintained. In radish taproots, the proteins of RsPIPs and RsTIPs account for 10% (Ohshima et al. 2001) and 40% (Higuchi et al. 1998) of the plasma and vacuolar membranes, respectively.

RsPIP2 group members in roots are responsive to salt and water stresses

Under water stress conditions especially in 0.3 M mannitol, the mRNA levels of the *RsPIP2* group members were increased 1 h after treatment and then decreased gradually (Fig. 1). However, this transient increase of the *RsPIP2-1* mRNA was not reflected in the protein amount of RsPIP2-1 (Fig. 5). The RsPIP2-1 protein level was gradually decreased after the treatment with mannitol or PEG. The reproducibility of the results during this study was confirmed by two or three independent experiments. There are some examples of dissimilarity between the changes in the mRNA and protein levels of aquaporins. In *A. thaliana*, drought stress caused a twofold increase in the PIP protein without an increment of the mRNA level (Kammerloher et al. 1994). The present result suggests a low translation rate and/or a rapid degradation of RsPIP2-1 protein in seedlings suffering water stress. The suppression of aquaporin level may be effective for reserving water in plant tissues, since water may be scattered and lost from roots under high osmotic conditions with mannitol and PEG.

In contrast, salt stress increased the level of RsPIP2-1 protein in roots. The amount of MIP-C protein was increased in *M. crystallinum* roots treated with 200 mM NaCl (Kirch et al. 2000). Some PIP genes of tobacco were increased in response to salt stress for several days (Yamada et al. 1997). In roots of

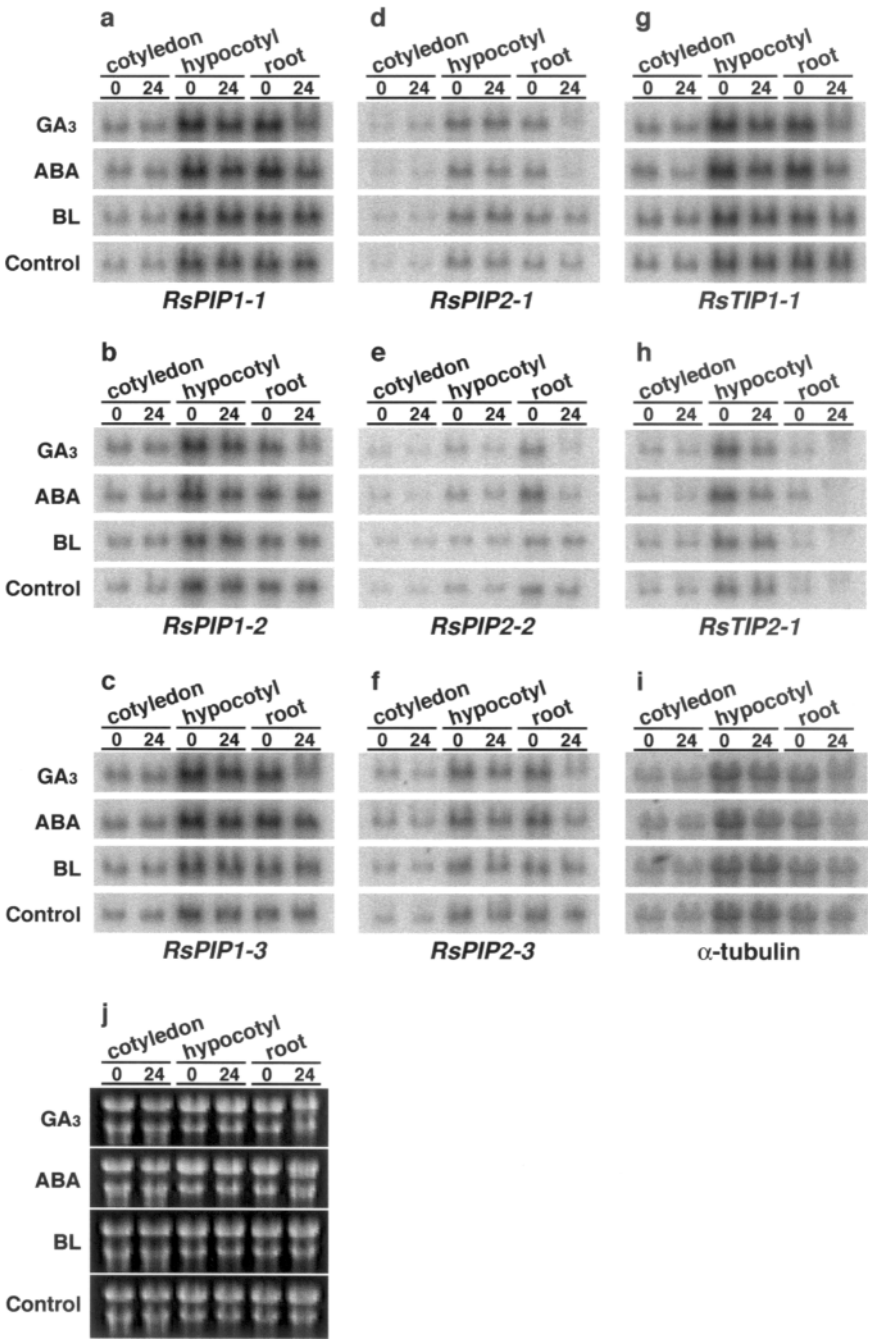


Fig. 6 Accumulation of aquaporin mRNAs in seedlings treated with phytohormones. Four-day-old seedlings were treated with 1 mM GA_3, 0.1 mM ABA, and 0.1 μM BL for 24 h. Total RNA was extracted from seedlings before (0) and after treatment (24) with phytohormones. Aliquots (10 μg) of RNA s were subjected to Northern blot analysis. (a) *RsPIP1-1*, (b) *RsPIP1-2*, (c) *RsPIP1-3*, (d) *RsPIP2-1*, (e) *RsPIP2-2*, (f) *RsPIP2-3*, (g) *RsTIP1-1*, (h) *RsTIP2-1*. (i) α-tubulin as a control. (j) rRNAs stained with ethidium bromide.

M. crystallinum (Yamada et al. 1995) and rice (Li et al. 2000) the mRNA level of PIPs was decreased transiently and then increased under salt stress conditions. An increase in the amount of RsPIP2-1 in radish and PIPs in other plants may be involved in the rapid uptake of water into cells to dilute NaCl that has entered into root cells. Furthermore, a rapid uptake of water and the increased root pressure may push up water with NaCl from roots to the other organs. This may effectively dilute NaCl in the plant body.

RsPIP2 group is responsive to ABA and GA_3

Gibberellic acids are well known for their influence on

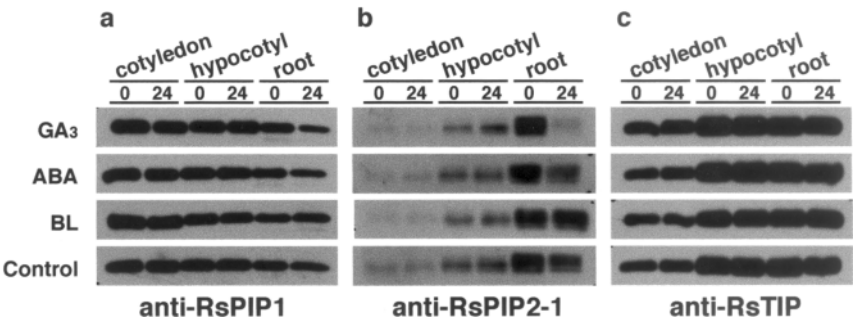


Fig. 7 Effects of phytohormones on the accumulation of aquaporin proteins in seedlings. Four-day-old seedlings were treated with GA_3, ABA and BL for 24 h. Crude microsomal membranes were prepared from seedlings before (0) and after (24) treatment with phytohormones, and then aliquots (5 μg) were subjected to SDS-PAGE and subsequent immunoblotting with antibodies to RsPIP1 (a), RsPIP2-1 (b) and RsTIP (c).

stem elongation, ABA for their growth inhibiting effect, and brassinosteroids for stimulation of stem elongation and proton pump activation (Hooykaas et al. 1999). In radish seedlings, BL, a kind of brassinosteroid, did not affect the level of aquaporins. ABA and GA_3 shifted the mRNA level of aquaporins downwards, especially *RsPIP2s* and *RsTIP1-1*. The amount of RsPIP2-1 protein decreased markedly, although the level was slightly decreased under the control condition. ABA and GA_3 enhanced this reduction. RsPIP2-1 protein was also decreased by osmotic stresses. There is a possibility that ABA and/or GA_3 are involved in the reduction of RsPIP2-1 protein by water stresses. In developing seeds, the mRNA level of *RsPIP2s* and *RsTIP1-1* were scarcely detected, although the mRNAs of *RsPIP1s* were accumulated at a high level (Suga et al. 2001). It is probable that the absence or suppression of *RsPIP2*s in developing seeds is mediated by the endogenous ABA.

Several groups have investigated the effects of phytohormones on aquaporins. Gene expression of *Arabidopsis PIP1b* (*PIP1-2*) (Kaldenhoff et al. 1993) and rice γ-*TIP* (*TIP1-1*) (Liu et al. 1994) was extensively enhanced by ABA. ABA increased the mRNA level of *Cp-PIPa* in *Craterostigma plantagineum*, although it did not change the *Cp-PIPc* mRNA level and decreased the *Cp-TIP* mRNA level (Mariaux et al. 1998). Treatment with GA_3 increased the mRNA levels of *TIP1-1* (Phillips and Huttly 1994) and *PIP1-2* (Kaldenhoff et al. 1996) in *Arabidopsis*. Morillon et al. (2001) reported that BL increased extensively the osmotic water permeability of protoplasts in *Arabidopsis* mutants, which lacked the ability to synthesize brassinosteroids. However, BL has no marked effect on aquaporin. It should be noted that the response to phytohormones varies among isoforms of aquaporins in any plant species. In growing radish plants, the level of RsPIP2-1 may be regulated by phytohormones. For example, RsPIP2-1 protein was abundant in young roots (10 d old) and the level was decreased during the growth of roots and disappeared from the mature taproots (Suga et al. 2001). This reduction of RsPIP2-1 level may be mediated by endogenous GA_3 that stimulates the taproot development, because a marked decrease in the RsPIP2-1 protein level occurred in seedlings treated with GA_3 (Fig. 7).

Aquaporins have been estimated to be involved in the cell elongation process in plants. Therefore, GA_3 that stimulates stem elongation in most cases has been estimated to induce expression and accumulation of aquaporins. However, GA_3 had an opposite effect in seedlings. Treatment with GA_3 did not stimulate the elongation of hypocotyls of 4-day-old seedlings. For seedlings, it has been assumed that GA_3 stimulates both the synthesis in the cell wall space and release of reactive oxygen intermediates such as H_2O_2 (Schopfer et al. 2001). Furthermore, Henzler and Steudle (2000) proposed that the aquaporin acts as a H_2O_2 channel. Assuming this hypothesis, the reduction of aquaporins by GA_3 added exogenously may suppress the transport of H_2O_2 into the cells. This hypothesis remains to be tested by biochemical experiments.

We believe that protein quantification is essential for understanding the physiological functions of aquaporins in situ. The level of mRNA was not directly reflected in the amount of translated product as demonstrated in this study. There are several factors that affect the level of protein expressed; namely the mRNA half-life, the translation rate, the rate of intracellular trafficking of membrane proteins, and the protein stability at the final destination.

In conclusion, the RsPIP2 group members, especially RsPIP2-1, are responsive to water stresses and phytohormones, while the RsPIP1 group members and RsTIP1-1 are constitutive aquaporins. The primary sequences are highly conserved among the RsPIP1 and RsPIP2 groups, respectively (Fig. 4). However, the sequence identity between the two types is less than 67%. The sequences are different between the two groups especially in the long hydrophilic N-terminal and the short C-terminal regions and the loop *e* exposed to the extracellular space (Suga et al. 2001). Thus characteristics of the physiological function and response to stresses of each type may be closely related to the dissimilarity in the primary structure. The physiological function of the PIP1 group members in plants is a matter of debate. Chaumont et al. (2000) reported that the maize PIP1 group (ZmPIP1a and ZmPIP1b) has no water chan-

nel activity in the *Xenopus laevis* oocyte expression system, although ZmPIP2 expressed the activity. Also, the PIP1 member, but not PIP2, of *Samanea saman* has been reported to have no water channel activity in the oocyte assay system (Moshelion et al. 2002). In the case of *Arabidopsis*, the PIP1 group members have been reported to increase the water permeability of the membrane when expressed in *Xenopus* oocytes (Kammerloher et al. 1994). We have just started to examine the differences in the water channel activity and the functional regulation of the RsPIP1 and RsPIP2 groups by using the yeast expression system.

Finally we should note that the quantitative regulation of aquaporins is an essential step for controlling the water flow and its pathway in plant tissues. In a Crassulacean-acid-metabolism plant *Graptopetalum paraguayense*, the protein contents of PIPs and TIPs were very low, 20% and 1%, respectively, of that of those in radish taproots. The low contents of aquaporins were reflected in the osmotic water permeability of the membranes (Ohshima et al. 2001). In *Mimosa*, the rapid water flux from vacuoles in motor cells has been demonstrated to be due to a large quantity of TIPs (Fleurat-Lessard et al. 1997). As the next step, we should quantify the amount of aquaporin isoforms in each cell of radish.

Materials and Methods

Plant materials

Radish (*Raphanus sativus* L. cv Tokinashi-daikon) seeds were germinated in the dark at 26°C. The standard medium contained 0.3 mM $CaSO_4$. Five-day-old seedlings were transferred to a solution containing 150 mM NaCl, 300 mM mannitol or 20% polyethylene glycol (PEG-3640, Sigma), and were grown in the dark at 26°C. In some experiments, 4-day-old seedlings were transferred to a solution that contained 0.1 mM ABA, 0.1 μM BL or 1 mM GA_3, and then incubated in the dark at 26°C for 24 h. Control seedlings were grown in water. The fresh weight of 4- and 5-day-old seedling was 80 and 90 mg per plant, respectively.

RNA extraction, DNA probe preparation and Northern blot analysis

Cotyledons, hypocotyls and roots of seedlings were frozen in liquid nitrogen and ground in a mortar with a pestle. RNA was extracted from the frozen powder of the tissue by the phenol-SDS extraction method (Suga et al. 2001). For Northern blot analysis, RNAs were electrophoresed in 1.0% agarose gel, and capillary transferred to a nylon membrane. The membrane was prehybridized in a hybridization buffer at 42°C for several h and was hybridized with a DNA probe (2×10^6 cpm ml^{-1}) at 42°C over night. DNA probes for mRNAs of *RsPIP1-1*, *RsPIP2-1*, *RsTIP1-1* and *RsTIP2-1* were prepared as described previously (Higuchi et al. 1998, Suga et al. 2001). The DNA probes of *RsPIP1-2* (218 bp), *RsPIP1-3* (219 bp), *RsPIP2-2* (249 bp) and *RsPIP2-3* (232 bp) containing the 3′-untranslated sequences were prepared by PCR using gene-specific primers; *RsPIP1-2* (forward, 5′-CAGGGGAATGTCTCTGACTCTG; reverse, 5′-GGACCATTCATCGGTGCTGC), *RsPIP1-3* (forward, 5′-GCAAGGGGGAATGTCTTTGTT; reverse, 5′-CATCGGTGCTGCTCTTGCTG), *RsPIP2-2* (forward, 5′-CCCCGAGTACACAAACATTCAC; reverse, 5′-GGAGCTGCGATAGCTGCATTT), *RsPIP2-3* (forward, 5′-CCCCCATCACACAAGCATTCAC; reverse, 5′-TCATTGGAGCCGCCATCGCTG). A cDNA fragment of α-tubulin of *A. thaliana* (1 kbp, *Eco*RI–*Hinc*II) was used as a control. The membrane was washed twice with 2× SSC (1× SSC: 0.15 M NaCl, 15 mM sodium citrate) containing 0.1% SDS at room temperature for 5 min, three times with 0.1× SSC containing 0.1% SDS at 42°C for 20 min, and once with the same buffer at 65°C for 20 min, and then was exposed to an imaging plate for 20 h. Then its radioimage was analyzed with a Fuji image analyzer BAS2500. Dot-blot hybridization of plasmid DNAs containing the aquaporin cDNA was carried out by the same procedure.

Quantification of absolute amount of RNA

Aquaporin cDNAs were linearized by treatment with *Xho*I. The sense RNA was made by in vitro transcription using T3 polymerase and a SP6/T7 transcription kit (Roche) as the standard RNA. Sample RNA was prepared from seedling hypocotyls and applied to Northern analysis together with the standard RNA. The signal intensity of blots was measured with a Fuji BAS 2500 and the amount of mRNA of aquaporin was calculated individually from the standard curve.

Preparation of crude microsomes

Radish tissues were frozen in liquid nitrogen and ground in a mortar with a pestle. The frozen powder was mixed with four volumes of a homogenizing medium that contained 0.25 M sorbitol, 2 mM EGTA, 10 mM APMSF (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride), 1% (w/v) polyvinylpyrrolidone-40 (Sigma), 2 mM DTT and 50 mM Tris-acetate, pH 7.5. The homogenate was filtered and centrifuged at 10,000×*g* for 15 min. The supernatant was centrifuged at 150,000×*g* for 30 min, and then the precipitate was suspended in 0.25 M sucrose, 1 mM DTT and 10 mM potassium phosphate buffer, pH 7.8, and was used as the crude microsomal membrane.

Immunoblot analysis

Antibodies to RsPIP1 and RsPIP2-1 (Suga et al. 2001) and to RsTIP (Maeshima 1992) were prepared previously. SDS-PAGE was carried out in 12% polyacrylamide gels. For immunoblotting, proteins in gels were transferred to a polyvinylidene difluoride membrane (Millipore) with a semidry blotting apparatus. The membrane was treated with 5% de-fatted milk and then with the primary antibody. The blots were rinsed and then visualized with horseradish peroxidase-coupled protein A and chemiluminescent reagents (Amersham, U.K.).

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