Plant Cell Physiol. 38(11): 1226-1231 (1997)
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Expression of Plasma Membrane Water Channel Genes under Water Stress in *Nicotiana excelsior*

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Deduced amino acid sequences encoded by the cDNAs related to the MIP gene family from *Nicotiana excelsior* were characterized. Phylogenetic characterization of the products of corresponding genes named *NeMip1, NeMip2,* and *NeMip3* strongly suggested that they are water channel proteins localized in the plasma membrane. Organ specificity of the gene expression was examined in leaves, roots, and reproductive organs. *NeMip1* was expressed in roots and reproductive organs; however, it was hardly detectable in leaves. Two other genes, *NeMip2* and *NeMip3*, were expressed in all of organs examined. mRNA accumulation from the genes was investigated in leaves under salt- and drought-stresses. The results demonstrated that mRNA accumulation from all three genes increased under salt- and drought-stresses within one day. However, they showed different accumulation patterns. In addition to their up-regulation under salt- and drought-stresses, daily changes in *NeMip2* and *NeMip3* mRNA accumulation was observed under unstressed conditions in leaves.

Key words: Aquaporin — MIP gene family — *Nicotiana excelsior* — Water channel — Water-stress.

The gene for MIP was first isolated from the bovine lens fiber (Gorin et al. 1984). Since then, a number of genes encoding MIP homologues have been isolated from bacteria, yeast, plants, and animals (Park and Saier 1996). Several members of the MIP gene family from animals and plants were experimentally proven to encode water specific channel proteins (aquaporins) (Daniels et al. 1994, Fushimi et al. 1993, Maurel et al. 1993, Preston et al. 1992, Yamada et al. 1995). Aquaporins form water specific pores in membranes so that water molecules can follow the gradient of osmotic pressures across the membrane. In plants, aquaporins have been found in the tonoplast and plasma membrane. γ-TIP from *Arabidopsis thaliana* was first documented as an aquaporin in plants (Maurel et al. 1993). The product of *RD28* (Yamaguchi-Shinozaki et al. 1992) was demonstrated as an aquaporin localized to the plasma membrane (PIP) (Daniels et al.1994). Genes for TIP and PIP comprise a large multi-gene family. Multiple PIP genes have been found in *Arabidopsis thaliana* (Kaldenhoff et al. 1993, Kammerloher et al. 1994, Yamaguchi-Shinozaki et al. 1992) and *Mesembryanthemum crystallinum* (Yamada et al. 1995). This gene multiplicity may reflect demands for the control of water permeability of membranes by differential gene expression and/or by specialized function. Expression of PIP genes is induced by desiccation in glycophytes (Fray et al. 1994, Guerrero et al. 1990, Yamaguchi-Shinozaki et al. 1992). On the other hand, in *M. crystallinum*, a facultative halophyte, the expression of aquaporin genes was down-regulated for the first 30 h after salt-stress with 400 mM NaCl and then recovered to the prestressed level (Yamada et al. 1995). When exposed to high salinity or drought conditions, most plants, either glycophytes or halophytes, begin to accumulate low molecular weight metabolites such as proline, glycine-betaine, or polyols in cells (Bohnert et al. 1995). These compounds maintain a high osmotic pressure in the cells so that the plants can continue to uptake water. Differences in the regulation of aquaporin gene expression between glycophytes and halophytes may reflect the importance of PIP gene regulation in stress tolerance in plants.

The *Nicotiana* species are not classified as halophytic plants, but some of their native habitats are harsh environments, either dry or salty. We screened 57 *Nicotiana* species for salt- and drought-tolerance (Komori, T. unpublished data). *Nicotiana excelsior* was found to be more tolerant of salt-stress than other *Nicotiana* species such as *Nicotiana tabacum* L. cv. BY4 or *Nicotiana rustica* (Komori, T., unpublished data). We are interested in knowing if the expression of aquaporin genes is related to stress tolerance in this species. As the first step in investigating the tolerance mechanism of *N. excelsior*, we isolated three MIP-related cDNAs (Yamada et al. 1997) and studied the expression of these genes under stress.

Materials and Methods

Plant materials—Plants (*Nicotiana excelsior*) were grown from seeds in a greenhouse. Seedlings were transferred to plastic pots (10 cm in diameter) filled with a mixture [1 : 1 (v/v)] of ver-

Abbreviation: MIP, major intrinsic protein; TIP, tonoplast intrinsic protein; PIP, plasma membrane intrinsic protein.

The nucleotide sequences reported in this paper have been submitted to the DDBJ/EMBL/GeneBank under the accession numbers AB002149 for *NeMip1* cDNA, AB002148 for *NeMip2* cDNA, and AB002147 for *NeMip3* cDNA.

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miculite and Hydroball (small particles of brick, purchased from a local supplier) three weeks after germination. Plants were watered daily with 50 ml of 25% Hoagland's solution (Hoagland's Solution No. 2, Sigma). They were grown in the greenhouse for an additional seven days, and then transferred to a growth chamber one week before stress was applied. The growth chamber was operated on a 12-h light (23°C)/12-h dark (20°C) cycle. Light was provided by 400 W metal halide lamps at a constant 500 to 600 $\mu E\ m^{-2}\ s^{-1}$. Salt-stress was imposed by daily application of 100 ml of 25% Hoagland's solution containing 250 mM NaCl. Watering was withheld in drought-stress treatment. Control plants were watered daily with 100 ml of 25% Hoagland's solution. Plants for the experiment examining organ specific expression were grown in the greenhouse from the sowing through the anthesis.

Deduced amino acid sequence analyses—Deduced amino acid sequence analyses, including phylogenetic study, of the NeMip products were done with GENETYX-MAC ver. 9.0 (Software Development Co. Ltd., Tokyo, Japan). Database searches were done with GENETYX-MAC/CD ver. 35.0 (Software Development Co. Ltd.).

RNA gel blot analysis—Total RNA was isolated from leaves of stressed plants and leaves, roots, or flowers of unstressed plants, as described previously (Ostrem et al. 1987). RNA gel blotting was done with 5 μg of LiCl-purified total RNA as described previously (Vernon and Bohnert 1992). Probes specific to each NeMip gene were prepared from 3′ untranslated regions of each cDNA and used for RNA gel blot analysis. Quantitation of signals was done with FUJX BAS1000 (Fuji Photo Film Co. Ltd., Tokyo, Japan).

DNA gel blot hybridization—Total DNA was isolated from leaves as described previously (Hiei et al. 1994). DNA gel blot hybridization was done with 20 μg of total DNA digested with restriction enzymes. The same probes used for RNA gel blot analysis were used for DNA gel blot analysis.

Results

Characterization of deduced amino acid sequences of NeMip1, NeMip2, and NeMip3—The three clones isolated, *NeMip1*, *NeMip2*, and *NeMip3*, had reading frames of 287, 287, and 285 amino acids, respectively. The identity of gene products at the amino acid level is 96.5% between *NeMip1* and *NeMip2*, 87.3% between *NeMip1* and *NeMip3*, and 88.2% between *NeMip2* and *NeMip3* (Yamada et al. 1997). Figure 1a shows the results of the homology analysis of the NeMip products with several PIP aquaporins from other plants (Höfte et al. 1992, Johansson et al. 1996, Kammerloher et al. 1994, Yamaguchi-Shinozaki et al. 1992). Proteins encoded by these genes contained the characteristic features of the MIP family; six putative membrane spanning regions (shown by solid bars) and two asparagine-proline-alanine (NPA) motifs (boxed) (Chrispeels and Agre 1994). The NeMip products did not contain the cysteine residue that confers mercury sensitivity to CHIP28 (Preston et al. 1993). The phosphorylation site of PM28A (Ser-274) (arrowed) (Johansson et al. 1996) was not presented in the NeMip products. The result of phylogenetic analysis (Fig. 1b) revealed that all of the gene products belong to the PIP1 subfamily.

Organ specificity of the NeMip gene expression—Organ specificity of the NeMip gene expression was investigated in leaves, roots, immature buds, mature buds, and flowers of *N. excelsior* (Fig. 2). Accumulation of *NeMip1* mRNA was almost equal in roots and floral organs, but very weak in leaves. Accumulations of the *NeMip2* mRNA and *NeMip3* mRNA were detectable in all organs examined. Level of *NeMip2* mRNA was lower in leaves than in other organs. *NeMip3* mRNA was five to ten times higher in leaves than in other organs.

Accumulation of NeMip mRNA under stress—Time courses of the mRNA levels of each NeMip gene after salt- or drought-stress were analyzed by RNA gel blot analysis, and their relative strengths were quantified by taking the strongest signal in each blot as unity (Fig. 3). In control plants, the mRNA levels of the NeMip genes were maintained at low levels. Moreover, after stress was imposed, their levels increased, but in different profiles. Under salt-stress, mRNA for *NeMip1* and *NeMip2* steadily increased, but the increase in *NeMip1* mRNA was greater than that in *NeMip2* mRNA. In contrast, the mRNA level of *NeMip3* rapidly increased within 24 hours after initiation of salt-stress and reached a plateau. The time course profiles of mRNA levels of the three genes after drought-stress were quite different from those after salt-stress. mRNA for *NeMip3* greatly increased within 24 hours after drought-stress and then decreased gradually, whereas mRNA for *NeMip2* increased steadily. Although mRNA for *NeMip1* moderately increased in the beginning, the level did not change thereafter. The mRNA levels of all three genes in salt- or drought-stressed plants remained significantly higher than in the control plants at the ends of five-day experimental periods.

Daily changes of NeMip mRNA accumulation—When RNA samples were extracted from the control plants at different times of day and subjected to RNA gel blot analysis, we noticed that steady-state levels of *NeMip2* and *NeMip3* mRNAs were high in the morning and low in the evening (Fig. 3); the change appeared cyclic. To examine this phenomenon in more detail, we carried out RNA blot analysis in leaves harvested at 8:15 a.m., 2:15 p.m., and 8:15 p.m. in the growth chamber for the three consecutive days. The illumination started at 8:00 a.m. and terminated at 8:00 p.m. Samples were harvested at the same time in the greenhouse. Accumulations of *NeMip2* mRNA and *NeMip3* mRNA were the highest at 8:15 a.m. and decreased as time passed (Fig. 4). This cycle was observed throughout the experimental period of three days. The fluctuation of mRNA levels was more remarkable in *NeMip3* than in *NeMip2*. This fluctuation was also observed in plants grown in the greenhouse under natural light.

Gene complexity of the three NeMip genes—Multiple bands with different signal strengths were observed after hybridization with probes specific for each NeMip gene to

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NEMIP1   1 MAENKEEDVKLGANKYRETQPLGTAAQTDKDYKEPPPAPLFEPGELSSWSFYRAGIAEFMATFLFLYITILTVMG------LKRSDSLCSSVGIQGVAWAF  95
NEMIP2   1 MAENKEEDVKLGANKFRETQPLGTAAQTDKDYKEPPPAPLFEPGELSSWSFYRAGIAEFMATFLFLYITILTVMG------LKRSDSLCSSVGIQGVAWAF  95
NEMIP3   1 M-ENKEEDVRLGANKYSERQAIGTAAQSDKDYTEPPPAPLFEAGELTSWSFYRAGIAEFMATFLFLYITILTVMG------VSKSESKCSTVGIQGIAWAF  94
PIP1a    1 M-EGKEEDVRVGANKFPERQPIGTSAQSDKDYKEPPPAPFFEPGELSSWSFWRAGIAEFIATFLFLYITVLTVMG------VKRSPNMCASVGIQGIAWAF  94
PIP1c    1 M-EGKEEDVRVGANKFPERQPIGTSAQTDKDYKEPPPAPFFEPGELSSWSFYRAGIAEFIATFLFLYITVLTVMG------VKRAPNMCASVGIQGIAWAF  94
PIP2a    1 M--AK--DV----------EAVPGEGFQTRDYQDPPPAPFIDGAELKKWSFYRAVIAEFVATLLFLYITVLTVIGYKIQSDTDAGGVDCGGVGILGIAWAF  87
PM28A    1 M--SK--EV----------SEEAQAHQHGKDYVDPPPAPFFDLGELKLWSFWRAAIAEFIATLLFLYITVATVIGHS-K-ET-V--V-CGSVGLLGIAWAF  81
RD28     1 M--AK--DV----------EG-P-DGFQTRDYEDPPPTPFFDAEELTKWSLYRAVIAEFVATLLFLYVTVLTVIGYKIQSDTKAGGVDCGGVGILGIAWAF  85
           *    *    *                **  *** *     **  **  ** **** ** **** *  ** *                  *  **  * ****

NEMIP1  96 GGMIFALVYCTAGISGGHINPAVTFGLFLARKLSLTRALFYMVMQCLGAICGAGVVKGFMVGPYQRLGGGANVVQPGYTKGDGLGAEIIGTFVLVYTVFSA  196
NEMIP2  96 GGMIFALVYCTAGISGGHINPAVTFGLFLARKLSLTRAIFYMVMQCLGAICGAGVVKGFMVGPYQRLGGGANVVNPGYTKGDGLGAEIIGTFVLVYTVFSA  196
NEMIP3  95 GGMIFALVYCTAGISGGHINPAVTFGLFLARKLSLTRAVFYMVMQCLGAICGAGVVKGFGKTLYQTKGGGANVVNHGYTKGSGLGAEIVGTFVLVYTVFSA  195
PIP1a   95 GGMIFALVYCTAGISGGHINPAVTFGLFLARKLSLTRALYYIVMQCLGAICGAGVVKGFQPKQYQALGGGANTVAHGYTKGSGLGAEIIGTFVLVYTVFSA  195
PIP1c   95 GGMIFALVYCTAGISGGHINPAVTFGLFLARKLSLTRAVFYIVMQCFGAICGAGVVKGFQPNPYQTLGGGANTVAHGYTKGSGLGAEIIGTFVLIYTVFSA  195
PIP2a   88 GGMIFILVYCTAGISGGHINPAVTFGLFLARKVSLPRALLYIIAQCLGAICGVGFVKAFQSSYYTRYGGGANSLADGYSTGTGLAAEIIGTFVLVYTVFSA  188
PM28A   82 GGMIFVLVYCTAGISGGHINPAVTFGLFLARKVSLLRALVYMIAQCLGAICGVGLVKAFMKGPYNQFGGGANSVALGYNKGTALGAEIIGTFVLVYTVFSA  182
RD28    86 GGMIFILVYCTAGISGGHINPAVTFGLFLARKVSLIRAVLYMVAQCLGAICGVGFVKAFQSSHYVNYGGGANFLADGYNTGTGLAAEIIGTFVLVYTVFSA  186
           ***** ****************************** ** **  *   ** ***** * ** *    *   *****   **  *  * *** ***** ******

NEMIP1 197 TDAKRNARDSHVPILAPLPIGFAVFLVHLATIPITGTGINPARSLGAAIIFNHDQAWDDHWIFWVGPFIGAALAAVYH-QIIIR-AIP----FKTKS--  287
NEMIP2 197 TDAKRNARDSHVPILAPLPIGFAVFLVHLATIPITGTGINPARSLGAAIIYNDEHAWDDHWIFWVGPFIGAALAAVYHSNNEQSYSIPQV--FLSLLK-  292
NEMIP3 196 TDAKRSARDSHVPILAPLPIGFAVFLVHLATIPITGTGINPARSLGAAIIYNQDHAWDDHWIFWVGPFIGAALAALYQ-QVVIR-AIP----FKSK---  285
PIP1a  196 TDAKRNARDSHVPILAPLPIGFAVFLVHLATIPITATGINPARSLGAAIIYNKDHSWDDHWVFWVGPFIGAALAALYH-VVVIR-AIP----FKSRS--  286
PIP1c  196 TDAKRSARDSHVPILAPLPIGFAVFLVHLASIPITGTGINPARSLGAAIIYNKDHAWDDHWIFWVGPFIGAALAALYH-QLVIR-AIP----FKSRS--  286
PIP2a  189 TDPKRSARDSHVPVLAPLPIGFAVFMVHLATIPITGTGINPARSFGAAVIYNKSKPWDDHWIFWVGPFIGAAIAAFYHQFVLRASGSKSLGSFRSAANV  287
PM28A  183 TDPKRSARDSHVPILAPLPIGFAVFMVHLATIPITGTGINPARSFGPAVIFNSNKVWDDQWIFWVGPFIGAAVAAAYHQYVLRAAAIKALGSFRSNPTN  281
RD28   187 TDPKRNARDSHVPVLAPLPIGFAVFMVHLATIPITGTGINPARSFGAAVIFNKSKPWDDHWIFWVGPFIGATIAAFYHQFVLRASGSKSLGSFRSAANV  285
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Fig. 1a

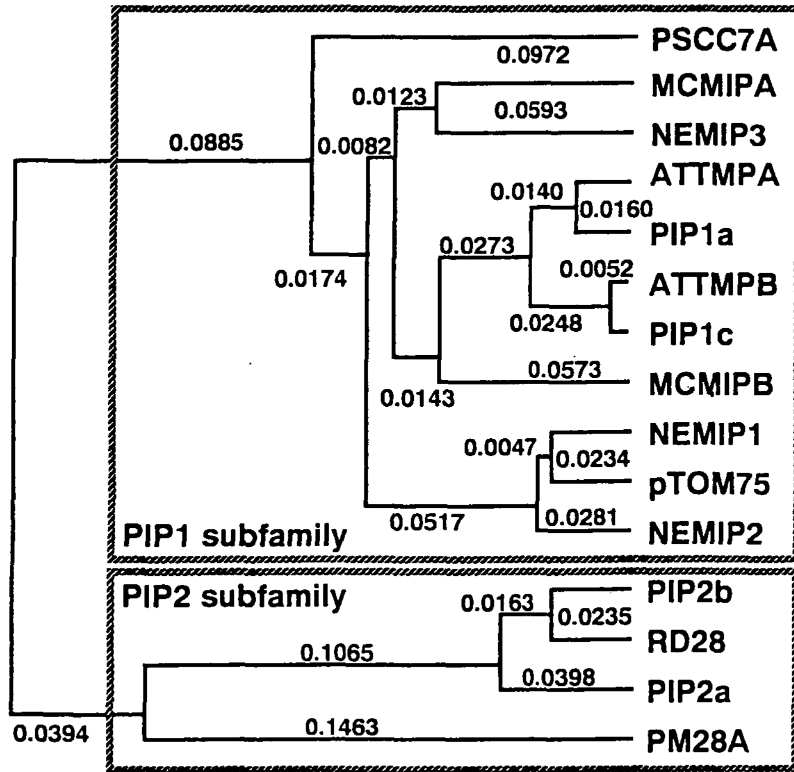

Fig. 1b

Fig. 1 (a) Comparison of amino acid sequences of the NeMips and other aquaporins. Two conserved NPA motifs are indicated by boxes. Putative transmembrane domains are indicated by solid bars above the alignment. Conserved amino acids are indicated by asterisks under the alignment. The phosphorylation site of PM28A (Ser-278) is indicated by an arrow. (b) The phylogenetic tree of plant PIPs. PIP1 and PIP2 subfamilies are enclosed in boxes. NEMIPA (AB002149), NEMIP2 (AB002148), and NEMIP3 (AB002147); PIP aquaporins in *N. excelsior*, MCMIPA (L36095) and MCMIPB (L36097); PIP aquaporins in *M. crystallinum*, ATTMPA (X68293), ATTMPB (X69294), PIP1a (X75881), PIP1c (X75882), PIP2a (X75883), PIP2b (X85774), and ATRD28 (D13254); PIP aquaporins in *A. thaliana*, PM28A (L77969); PIP aquaporin in *Spinacia oleracea*, pTOM75 (X73848); PIP aquaporin in *Lycopersicon esculentum*.

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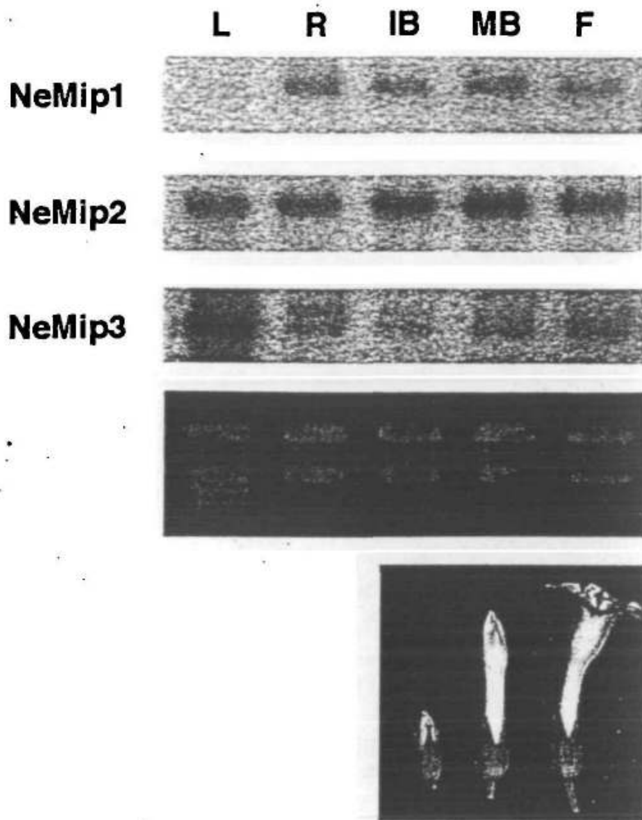
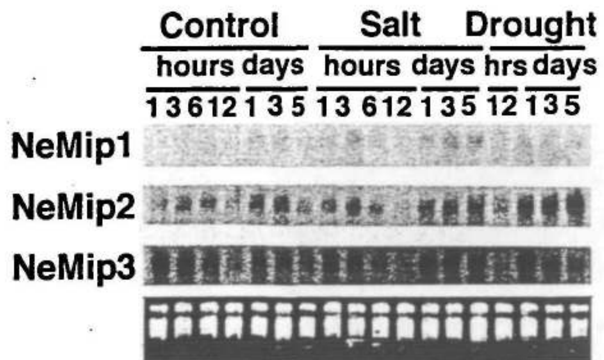


Fig. 2 Northern blot analysis of the NeMip genes for tissue specificity. Total RNA was extracted from indicated tissues of *N. excelsior*. Five μg of LiCl-purified RNA per lane was resolved on a formaldehyde agarose gel and blotted onto a nylon membrane. Northern hybridization was done with probes specific to each NeMip gene. L, leaf; R, root; IB, immature bud; MB, mature bud; F, flower.



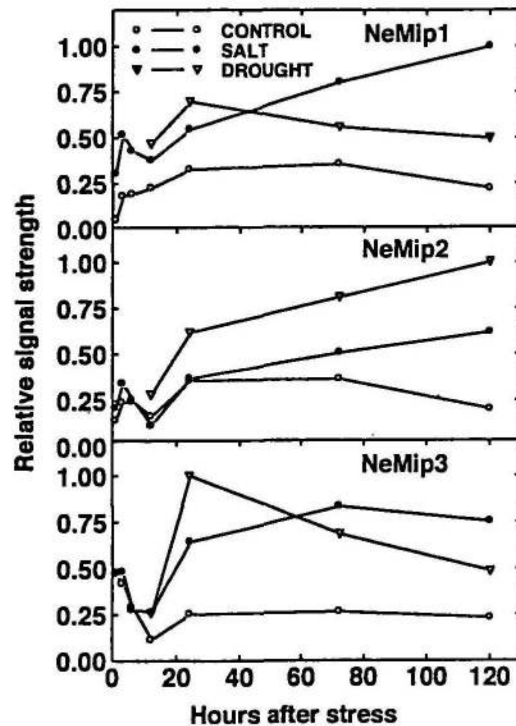


Fig. 3 Northern blot analysis of the NeMip genes for stress response. Total RNA was extracted from leaf tissue of *N. excelsior* at the indicated times after stress treatments. The treatments were started at 8:00 a.m. Five μg of LiCl-purified RNA per lane was resolved on a formaldehyde agarose gel and blotted onto a nylon membrane. Northern hybridization was done with probes specific to each NeMip gene. The result was quantified by FUJX BAS1000, and the strongest signals in each blot are taken as unity (lower panel).

total *N. excelsior* DNA (Fig. 5). This result indicates that each gene may include a few closely related genes.

Discussion

A number of genes belonging to the MIP family have been isolated from animals, bacteria, yeast, and plants (Park and Saier 1996). Even though some of their functions are not yet known, several of them were experimentally proven to be aquaporins in animals (Fushimi et al. 1993) and plants (Daniels et al. 1994, Maurel et al. 1993, Yamada et al. 1995). A phylogenetic study among genes within the MIP family revealed that it was composed of at least five subfamilies; bacterial glycerol (or metabolites) facilitators, Nod-MIP, plant PIP, plant TIP, and mammalian aquaporins (Yamada et al. 1995). PIP can be further categorized into PIP1 and PIP2 (Kammerloher et al. 1994). Several PIP1, PIP2, and TIP genes are known to encode aquaporins (Daniels et al. 1994, Maurel et al. 1993, Yamada et al. 1995). The products of NeMips studied in this paper were all classified in the subfamily of PIP1, based on a phylogenetic study (Fig. 1b).

The promoter of an aquaporin gene in *M. crystallinum* is active in immature flowers in transgenic tobacco, but less active in mature flowers (Yamada, S. unpublished data). In *N. excelsior*, however, mRNAs of the three NeMip genes were detected at a certain level in immature as well as

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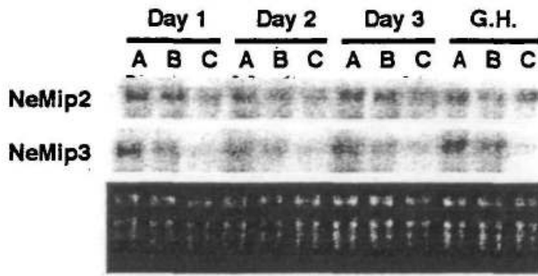

Fig. 4 Northern blot analysis of *NeMip2* and *NeMip3* for mRNA accumulation changes. Total RNA was extracted from leaf tissue of *N. excelsior* grown in the growth chamber or in the greenhouse (G.H.) at the indicated times. Five μg of LiCl-purified RNA per lane was resolved on a formaldehyde agarose gel and blotted onto a nylon membrane. Northern hybridization was done with probes specific to each NeMip gene. A, 8:15 a.m.; B, 2:15 p.m.; C, 8:15 p.m.

mature flowers, although the relative levels among the three genes differed (Fig. 2).

The expression profiles in leaves of the NeMip genes and of the Mip genes in the common ice plant also differed. The NeMip genes and several other PIP genes in glycophytes are up-regulated under water stress (Fray et al. 1994, Guerrero et al. 1990, Yamaguchi-Shinozaki et al. 1992). In contrast, in the common ice plant, a facultative halophyte, the mRNA level of PIP first decreased during 6 to 30 h after water stress, but resumed to or exceeded the pre-stress level (Yamada et al. 1995). This profile correlated with the time course of turgor transition in the whole plant. In *N. excelsior* which originated from the central part of Australia and was selected as a salt-tolerant species by screening of

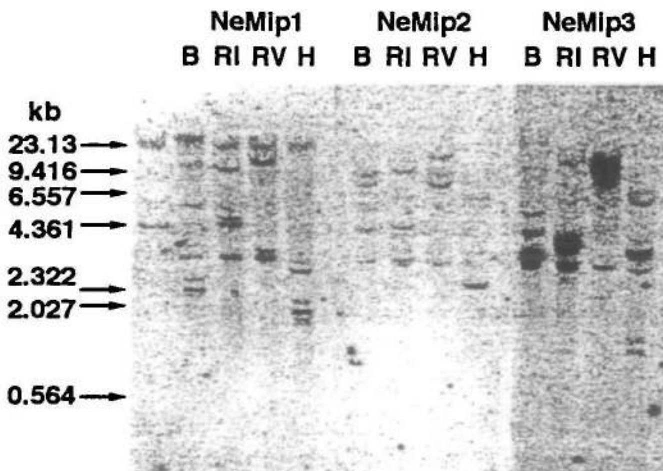

Fig. 5 DNA gel blot analysis of the NeMip genes for gene complexity. Twenty μg of genomic DNA was digested with the restriction enzyme indicated and subjected to agarose gel electrophoresis. DNA was blotted onto a nylon membrane and hybridized with probes specific to each NeMip gene. B, *Bam*HI; RI, *Eco*RI; RV, *Eco*RV; H, *Hin*dIII.

the *Nicotiana* species (Komori, T. unpublished data), the expression profiles of the NeMip genes is much more complicated. The three NeMip genes studied in this work are up-regulated after salt- and drought-stresses, but the time courses of their expression are not the same. mRNA levels of *NeMip1* and *NeMip3* changed with characteristic profiles under salt- and drought-stresses; however, those of *NeMip2* increased in a similar manner under both salt- and drought-stresses. It appears that *NeMip2* gene responds generally to stress. The changes in *NeMip3* mRNA levels likely associate with the water status of leaves. On the first day of salt application, the decrease in water potential was only slightly greater than the control; then a drastic decrease was observed after two days of salt- or drought-stress (Komori, T. unpublished data), and the expression profile of *NeMip3* was found to be more related to the water status in leaves than that of *NeMip2*. From this fact, we presume that *NeMip3* plays a role in maintaining the water status under stress. In our system, stresses are first applied to root system. Therefore, changes in mRNA level of the NeMip genes in the root of *N. excelsior* are expected to provide more informative results. Unfortunately, however, our culture method was not suitable for collecting intact roots from stressed plants, and the use of hydroponic culture is under way to cope with the problem.

Desiccation responsive MIP-related genes have been reported in pea (*Trg31*) (Guerrero et al. 1990, Jones and Mullet 1995), *A. thaliana* (*RD28*) (Yamaguchi-Shinozaki et al. 1992), and tomato (*pTOM75*) (Fray et al. 1994). Although the products of *Trg31* (PSCC7A in Fig. 1b) and *pTOM75* have not yet been proven to be an aquaporin, they belong to the PIP1 subfamily (Fig. 1b). The product of *RD28* was proven to be an aquaporin (Daniels et al. 1994) and belongs to the PIP2 subfamily.

No salt-stress responsive PIP aquaporin gene has been reported in glycophytes. We demonstrated that the NeMip genes responded to salt-stress as well as drought-stress. The NeMip genes responded to salt- and drought-stresses differently, and each NeMip gene responded to waterstress differently (Fig. 3). Differential regulation in gene expression and the multiplicity of PIP genes in *N. excelsior* lead us to presume that individual PIP isogenes must be precisely regulated under stressed conditions and probably under unstressed conditions as well. By having multiple genes that can be expressed differently and thus respond to environmental changes differently, plants may maintain their sound water status.

Daily cyclic changes in mRNA levels for *NeMip3* and *NeMip2* were found in leaves but not in roots or flowers. Moreover, the cyclic changes in *NeMip2* mRNA were observed clearly under growth chamber conditions but not under greenhouse conditions, whereas the changes in *NeMip3* mRNA occurred clearly under both conditions. It is, therefore, unlikely that these daily cyclic changes in mRNA

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levels are circadian rhythms. It is more likely that they are associated with the water status of leaves. In *N. excelsior*, the water potential of leaves showed a similar daily change, being highest in the morning and gradually decreasing until evening (Komori, T. unpublished data). These daily changes in water potential correlated with those of the steady-state levels of *NeMip3* mRNA. It is assumed that the regulation of PIP gene expression is two phasic in response to water status: a moderate decrease in leaf water potential results in down-regulation, but the more drastic decrease as found after two days of salt- or drought-stress results in up-regulation. The results of DNA gel blot analysis indicated that *N. excelsior* has more PIP isogenes. Investigations of tissue specificity and expression regulation for other genes should clarify relationship between PIP aquaporins and water status in plants.

We wish to thank Yoshiko Amma for her technical assistance. This work has been supported by the Petroleum Energy Center of Japan.

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(Received May 2, 1997; Accepted September 3, 1997)

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