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MIP Genes are Down-regulated Under Drought Stress in *Nicotiana glauca*

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Water flux across cell membranes has been shown to occur not only through the lipid bilayer, but also through aquaporins, which are members of the major intrinsic protein (MIP) super-family of channel proteins. Aquaporins greatly increase the membrane permeability for water, but may also be regulated, allowing cellular control over the rate of water influx/efflux. Water flux is crucial for stomatal opening and closing, but little is known about the role that aquaporins play in stomatal physiology. Our initial goal was to isolate and characterize the *MIP* genes expressed in guard cells of the model plant, *Nicotiana glauca*. Degenerate oligonucleotides corresponding to amino acid sequences conserved in tonoplast intrinsic proteins (TIPs) or plasma membrane intrinsic proteins (PIPs) were used to amplify portions of *MIP* genes by RT-PCR. These PCR products were used as probes in screening a *N. glauca* guard cell cDNA library. We isolated three clones (*NgMIP1*, *NgMIP2* and *NgMIP3*) homologous to TIPs and two clones (*NgMIP4* and *NgMIP5*) homologous to PIPs. All of the *MIP* genes we characterized displayed highest levels of mRNA accumulation in roots or stems, with lower levels of expression in mesophyll cells and whole leaves, and lowest transcript accumulation in guard cell RNA. Interestingly, the accumulation of transcripts arising from *NgMIP2*, *NgMIP3* and *NgMIP4* diminished dramatically in drought-stressed plants. This down-regulation of *MIP* gene expression may result in reduced membrane water permeability and may encourage cellular water conservation during periods of dehydration stress.

Key words: Aquaporin — Gene expression — Plasma membrane intrinsic protein — Tonoplast intrinsic protein — Tree tobacco — Water channel protein.

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

The nucleotide sequences reported in this paper have been submitted to GenBank, EMBL and DDBJ databases and will appear under accession numbers AF290617 (*NgMIP1*), AF290618 (*NgMIP2*), AF290619 (*NgMIP3*), AF290620 (*NgMIP4*) and AF290621 (*NgMIP5*).

Introduction

The rate of water flux into or out of a cell is determined by the water potential gradient that acts as the driving force for transport and by the water permeability of the membrane. Aquaporin proteins facilitate osmosis by forming water-specific pores as an alternative to water diffusion through the lipid bilayer, thus increasing the water permeability of the membrane (Schäffner 1998, Kjellbom et al. 1999). In plants, aquaporins localized in the tonoplast are called tonoplast intrinsic proteins (TIPs), while those in the plasma membrane are PIPs (Maurel 1997). Evidence is accumulating that aquaporins do function in plants in osmoregulation and long-distance transport (Maggio and Joly 1995, Kaldenhoff et al. 1998). It is also known that aquaporin activity may be regulated by phosphorylation (Johansson et al. 1998).

The presence of aquaporins in plant cell membranes has inspired a re-evaluation of the mechanisms and regulation of water flux through tissues (Steudle and Henzler 1995). Stomatal function is dependent upon water flux across guard cell membranes to effect stomatal opening and closure. Little is known about the role that aquaporins might play in facilitating water flux across guard cell membranes or if osmotic water permeability of guard cell membranes changes under different conditions. It has been demonstrated that aquaporin genes are expressed in guard cells of sunflower (TIPs) (Sarda et al. 1997) and *Arabidopsis thaliana* (PIPs) (Kaldenhoff et al. 1995). One goal of this study was to identify aquaporin genes that are expressed in guard cells of *Nicotiana glauca*, a model plant for the study of stomatal physiology and guard cell gene expression (Dodge et al. 1992, Smart et al. 2000).

Aquaporins are members of a larger super-family of proteins, the major intrinsic proteins (MIPs), which includes channels that are permeable for glycerol and/or urea (Pao et al. 1991). In *A. thaliana*, the MIPs, including TIPs and PIPs, are encoded by a family of at least 30 genes that display differential patterns of expression. In general, MIPs are most abundantly expressed in rapidly growing tissues and in cells involved in high-volume water flux (Weig et al. 1997, Kjellbom et al. 1999). Using PCR-based cloning strategies and by screening a guard cell cDNA library, this work was aimed at determining whether there might be an aquaporin gene family member that is predominantly expressed in guard cells and might have evolved to serve a specialized function in stomatal physiology.

There is good evidence that aquaporins are involved in plant response to dehydration stress. The activity of aquapor-

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Table 1 Degenerate oligonucleotides used for PCR amplification of MIP sequences

Name	DNA sequence [a]	Orientation [b]	Corresponding amino acid sequence
LBS103	5′-AAYCCIGCIGTIACITTYGG-3′	S	NPAVTFG
LBS104	5′-ATRTTIGCICCIACDATRAA-3′	AS	FIVGANI
LBS116	5′-GGICCIACCCARWAIATCCA-3′	AS	WI(Y/F)WVGP
LBS117	5′-GCITGGGCITTYGGIGGIATGAT-3′	S	AWAFGGMI

[a] Single-letter codes: I = inosine; Y = C or T; R = A or G; D = A or G or T; W = A or T.
[b] Orientation of the oligonucleotide relative to corresponding amino acid sequence: S, sense; AS, antisense.

ins can be regulated by phosphorylation, and in spinach, the PM28A plasma membrane aquaporin is phosphorylated and active only under high water potential conditions (Maurel et al. 1995, Johansson et al. 1998). This aquaporin may be dephosphorylated and inactivated under conditions of dehydration stress, perhaps to allow cellular water conservation (Johansson et al. 1996, Johansson et al. 1998). In contrast, there are examples of aquaporin genes whose expression is induced by dehydration stress, which should result in greater osmotic water permeability and facilitated water flux (Yamaguchi-Shinozaki et al. 1992, Yamada et al. 1997, Sarda et al. 1999). Here we present data that in *N. glauca*, a plant that is well-adapted to arid climates, *MIP* gene expression is down-regulated under drought stress.

Materials and Methods

Plant material and growth conditions

N. glauca (Graham) seeds were the generous gift of Dr. Gary Tallman, Willamette College, who collected them from plants growing on or around the campus of Pepperdine University, Malibu, CA, U.S.A. Plants were grown in a 4 : 1 mixture of MetroMix 510 (Scotts, Marysville, OH, U.S.A.) and perlite in a growth chamber at 23–25°C under a regime of 12 h light (approximately 200 μmol m^{-2} s^{-1}) each day. Plants were watered every other day and fertilized once each week. Drought stress was applied to plants that were approximately 0.5 m tall by withholding water, and tissue was harvested for RNA isolation when the leaves were wilted, usually after 3–4 d.

Guard cell purification and nucleic acid isolation

RNA was isolated from highly enriched preparations of guard cells from leaves of either well-watered or drought-stressed plants as previously described (Smart et al. 1999). RNA was isolated from enriched preparations of mesophyll cell extract as described (Smart et al. 2000). A modified hot borate method was used to isolate RNA from roots, stems and de-veined leaves (Wan and Wilkins 1994). RNA preparations were completed in duplicate for each tissue. Genomic DNA was isolated from de-veined leaves essentially as previously described (Bernatzky and Tanksley 1986).

PCR amplification and cloning

Degenerate oligonucleotides were designed to conserved regions of TIPs and PIPs (Höfte et al. 1992, Weig et al. 1997) (Table 1) and were purchased from Genset (La Jolla, CA, U.S.A.). Primers LBS103, LBS104 and LBS116 were designed to regions conserved in nearly all types of plant MIPs, while LBS117 corresponds to a sequence found in plant PIPs, but not in TIPs (Kammerloher et al. 1994). PCR was performed using AmpliTaq DNA polymerase as described by the manufacturer (PE Applied Biosystems, Foster City, CA, U.S.A.). The templates were either a portion of a *N. glauca* guard cell cDNA library (Smart et al. 2000) or first-strand cDNA generated by reverse transcription of *N. glauca* guard cell RNA using SuperScript reverse transcriptase (Gibco BRL, Bethesda, MD, U.S.A.). PCR cycles were: 1 cycle of 94°C for 6 min; 35 cycles of 94°C for 1 min, 42°C or 48°C for 1.5 min and 72°C for 2 min; then a final cycle of 72°C for 7 min. PCR products were subcloned either by ligation into pCRII (Invitrogen, Carlsbad, CA, U.S.A.) or by topoisomerase reaction into pCR2.1 TOPO (Invitrogen) and the resulting plasmids used to transform *Escherichia coli* XL1-Blue (Stratagene, La Jolla, CA, U.S.A.). Plasmid DNA isolation and other DNA manipulations were performed using standard procedures (Ausubel et al. 1999).

Hybridization conditions

Probes for hybridizations were generated by first purifying the cloned inserts from plasmid DNA, then labeling those restriction fragments with [α-^{32}P]dATP by the random primer labeling method (Ausubel et al. 1999). Restriction digests of genomic DNA were resolved in 0.8% agarose gels and transferred to supported nitrocellulose (Schleicher and Schuell, Keene, NH, U.S.A.). RNA was denatured, stained with ethidium bromide, and resolved in 1.2% agarose-formaldehyde gels, which were photographed prior to being transferred to supported nitrocellulose. Hybridizations of Southern blots were performed in aqueous solution at 65°C, while plaque lift filters were hybridized in 50% formamide solution at 42°C (Smart et al. 2000) and Northern blots were hybridized in PerfectHyb Plus solution at 68°C as described by the manufacturer (Sigma). Final washes of filters were in 0.1× SSC (1× SSC is 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0) at 68°C. Hybridization was detected either by exposure to XAR-5 X-ray film (Eastman Kodak, Rochester, NY, U.S.A.) or by quantification with a Phosphorimager SI (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

DNA sequencing and analysis

Complete DNA sequence from both strands of the cloned inserts was obtained using either a LI-COR model 4200 (Lincoln, NE, U.S.A.) or an ABI model 377 (Foster City, CA, U.S.A.) automated sequencer. Sequence analysis was performed using the software package DNASTAR (Madison, WI, U.S.A.) and the program PSORT version 6.4 (http://psort.nibb.ac.jp). Multiple sequence alignment was performed using Clustal X (Thompson et al. 1997) and phylogenetic analysis was performed using PAUP version 4.0b4a for Macintosh (Swafford 2000). A copy of the sequence alignment is available upon request.

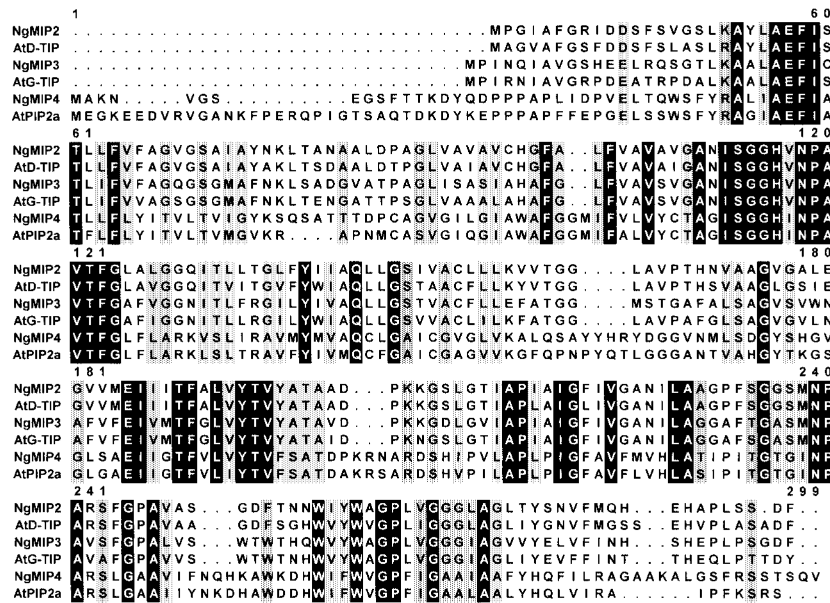

Fig. 1 Multiple sequence alignment of MIP sequences from *N. glauca* and *A. thaliana*. Deduced amino acid sequences were aligned using Clustal X. Residues that are identical in all sequences are shaded with black, while residues that are similar in all sequences are shaded with gray. The sequences of *N. glauca* NgMIP2 (AF290618), NgMIP3 (AF290619) and NgMIP4 (AF290620) were aligned with *A. thaliana* δ-TIP (U39485), γ-TIP (X72581) and PIP2a (X75883).

Results

Cloning of MIP genes expressed in guard cells

Both PCR amplification and cDNA library screening were used to isolate *MIP* genes expressed in guard cells of *N. glauca*. Initially, degenerate oligonucleotides designed to sequences common to most TIPs (LBS103 and LBS104, Table 1) were used to PCR-amplify portions of *MIP* genes from reverse transcribed guard cell RNA. This strategy yielded fragments representing two different genes, *NgMIP1* and *NgMIP2*. The fragment of *NgMIP2* was used as a probe in screening a guard cell cDNA library at medium stringency, and 76 positive plaques were isolated after the first round. Eighteen of these were purified and further characterized by hybridization and partial sequencing. Most of the clones obtained by this hybridization strategy represented *NgMIP2*, the gene used as the probe, while a few corresponded to another *MIP* gene, *NgMIP3*. The longest cDNAs were fully sequenced to reveal that the *NgMIP3* cDNA contained a complete open reading frame, while the longest *NgMIP2* cDNA isolated from the library was incomplete at the 5′-end and lacked sequence corresponding to the first several codons. A cDNA clone containing the 5′-end of the *NgMIP2* message was later obtained by PCR-amplification from the cDNA library using combinations of clone-specific primers and primers anchored in the λZAPII vector. The sequences of *NgMIP1* and *NgMIP2* are most similar to known δ-TIPs, while *NgMIP3* is most similar to γ-TIPs.

In order to isolate clones of putative *PIP* genes, degenerate oligonucleotides were designed corresponding to sequences conserved in PIPs (LBS117 and LBS116, Table 1). This primer pair was used to PCR-amplify fragments of two genes with sequence homology to *PIP*s, *NgMIP4* and *NgMIP5*. The fragment of *NgMIP4* was used as a probe to screen the guard cell cDNA library, and a cDNA clone containing the entire coding region of *NgMIP4* was obtained and sequenced.

Structure and phylogenetic analysis of MIP genes from N. glauca

The amino acid sequences deduced from the three full-length cDNAs included the features typical of MIP proteins (Fig. 1). The products of *NgMIP2*, *NgMIP3* and *NgMIP4* are predicted to be 25.0, 25.7 and 30.4 kDa, respectively, and each contains all of the residues conserved in all MIPs, including the two hallmark Asn-Pro-Ala sequence repeats (Chrispeels and Maurel 1994). All three are also predicted to form six membrane-spanning domains, but apparently lack N-terminal cleavable signal sequences based upon PSORT prediction. NgMIP2 and NgMIP3 are predicted to contain Cys residues (Cys116 in NgMIP2, Cys118 in NgMIP3) that were shown to confer Hg-sensitivity in Arabidopsis γ-TIP and δ-TIP (Daniels et al. 1996). NgMIP3 is predicted to contain the Ser residue (Ser276) that is phosphorylated in spinach PM28A (Johansson et al. 1998).

The sequences of the three full-length *N. glauca* cDNAs each represent members of different subfamilies of *MIP* genes. The predicted amino acid sequence of NgMIP2 is 83% identi-

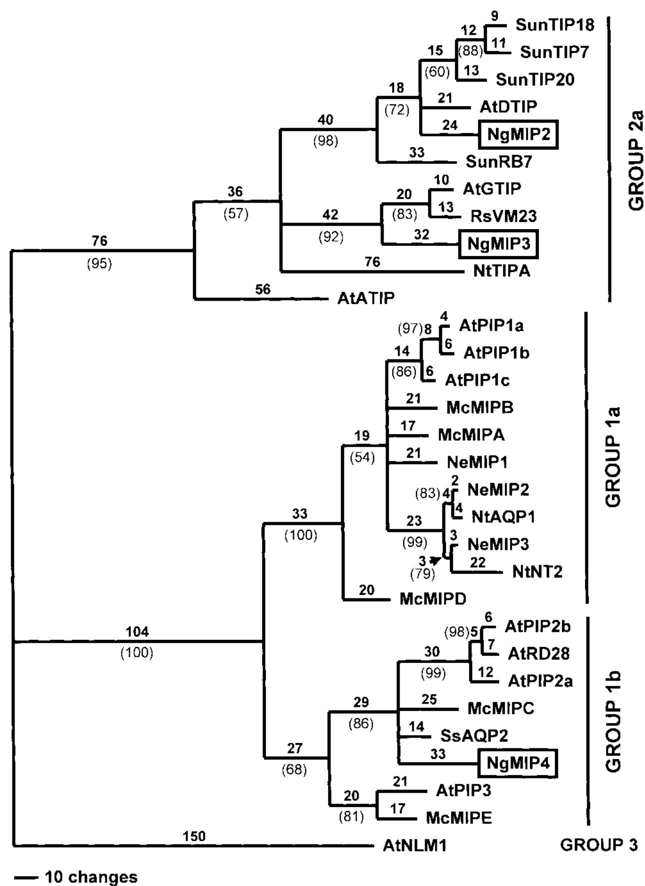

Fig. 2 Consensus phylogram of MIP protein sequences produced by maximum parsimony analysis using PAUP version 4.0b4a. Phylogenetic subgroups defined by Tyerman et al. (1999) are noted on the right. Bold numbers above each branch indicate branch lengths, while numbers in parentheses are bootstrap values from 1,000 bootstrap replicates. Starting trees were obtained by stepwise addition with simple addition sequence. The branch swapping algorithm was tree-bisection-reconnection. AtNLM1 was set as the outgroup. Sequences were aligned using Clustal X. Sequence sources were: *Helianthus annuus*, SunTIP18 (X95951), SunTIP7 (X95950), SunTIP20 (X95952) and SunRB7 (X95953); *A. thaliana*, AtDTIP (U39485), AtGTIP (X72581), AtATIP (X63551), AtPIP1a (X75881), AtPIP1b (Z17424), AtPIP1c (X75882), AtPIP2a (X75883), AtPIP2b (X75884), AtRD28 (D13254), AtPIP3 (U78297) and AtNLM1 (Y07625); *Raphinus sativus*, RsVM23 (D84669); *N. tabacum*, NtTIPA (AJ237751), NtAQP1 (AF024511) and NtNT2 (U62280); *M. crystallinum*, McMIPA (L36095), McMIPB (L36097), McMIPC (U73466), McMIPD (U26537) and McMIPE (U73467); *N. excelsior*, NeMIP1 (AB002149), NeMIP2 (AB002148) and NeMIP3 (AB002147); *Samanea saman*, SsAQP2 (AF067185); *N. glauca* (this paper), NgMIP2 (AF290618), NgMIP3 (AF290619) and NgMIP4 (AF290620).

cal to δ-TIP from Arabidopsis (Daniels et al. 1996), but is most like (86% identical) a putative δ-TIP from cotton (*Gossypium hirsutum*) fibers (GenBank accession number U62778). The predicted protein sequence of NgMIP3 is 77% identical to γ-TIP from Arabidopsis (Höfte et al. 1992, Maurel et al. 1993) and is very similar (99% identical) to a putative TIP from *Nicotiana tabacum* (GenBank accession number Y08161). The amino acid sequence of NgMIP4 is very similar to those of RD28 (79% identical) and PIP2a (81% identical) from Arabidopsis (Yamaguchi-Shinozaki et al. 1992, Kammerloher et al. 1994), but is most like (87% identical) a putative PIP from pulvini of *Samanea saman* (GenBank accession number AF067185). The deduced amino acid sequences from the partial-length PCR products of *NgMIP1* and *NgMIP5* have high identity to Arabidopsis aquaporins δ-TIP and RD28, respectively. The sequences of NgMIP1 and NgMIP2, both putative δ-TIPs, are 88% identical over the region of overlap (88 amino acids), while NgMIP4 and NgMIP5, both putative PIP2s, are 86% identical over their 156 residue overlap. We did not identify homologs of Arabidopsis α-TIP, NLM1, PIP1 or PIP3 (Weig et al. 1997) through our PCR-based approach or by library screening, although there were more than 50 hybridizing clones that were not characterized.

Phylogenetic analysis of the *N. glauca* MIP protein sequences was performed together with confirmed aquaporin sequences from Arabidopsis, sunflower and *Mesembryanthemum crystallinum*, as well as other related MIP sequences. This analysis suggests that NgMIP2, NgMIP3 and NgMIP4 are representatives of two of the six subgroups of plant aquaporins (Fig. 2) defined by Tyerman et al. (1999). NgMIP2 is an ortholog of Arabidopsis δ-TIP and aquaporins expressed in guard cells of sunflower (SunTIP7 and SunTIP20) (Sarda et al. 1997), while NgMIP3 is an ortholog of Arabidopsis γ-TIP and the radish VM23 tonoplast aquaporin (Higuchi et al. 1998). Finally, NgMIP4 is an ortholog of the subfamily of plasma membrane aquaporins that includes Arabidopsis PIP2 and RD28 and *M. crystallinum* MipC (Yamaguchi-Shinozaki et al. 1992, Daniels et al. 1994, Kammerloher et al. 1994). As in previous phylogenetic analyses, the sequences from *N. glauca* are more similar to orthologous genes from distantly related species than they are to sequences from other *Nicotiana* species, including *Nicotiana excelsior* and *N. tabacum* (Yamada et al. 1995, Tyerman et al. 1999).

Genomic organization of MIP genes in N. glauca

We have isolated cDNA clones originating from five different *MIP* genes, but genomic Southern analysis (Fig. 3) suggests that, as in other plants, there are additional *MIP* genes in *N. glauca*. Each of these clones hybridized strongly to one or sometimes two bands in each digest, but in many cases there was weak hybridization to as many as three additional bands in each digest. It was expected that *NgMIP1* and *NgMIP2* might cross-hybridize, since they are 84% identical over their region of overlap, and weak bands of similar sizes as those in the *NgMIP1* Southern hybridization are present in some lanes of the *NgMIP2* Southern (Fig. 3). Also, *NgMIP4* and *NgMIP5* are 76% identical over their region of overlap and might be expected to cross-hybridize.

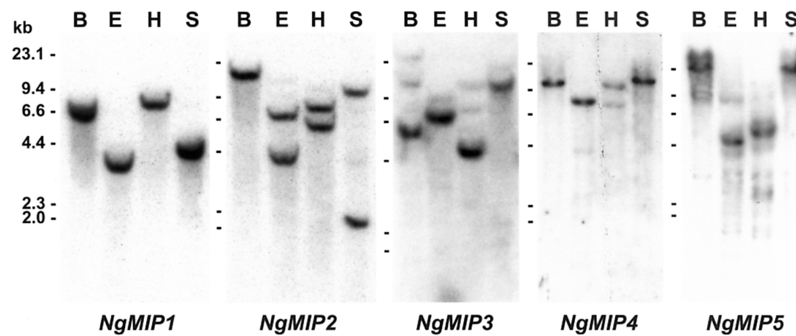

Fig. 3 Southern blots of *N. glauca* genomic DNA probed with clones of *NgMIP1*, *NgMIP2*, *NgMIP3*, *NgMIP4* and *NgMIP5*. DNA (20 µg) was digested with: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; or S, *Sac*I.

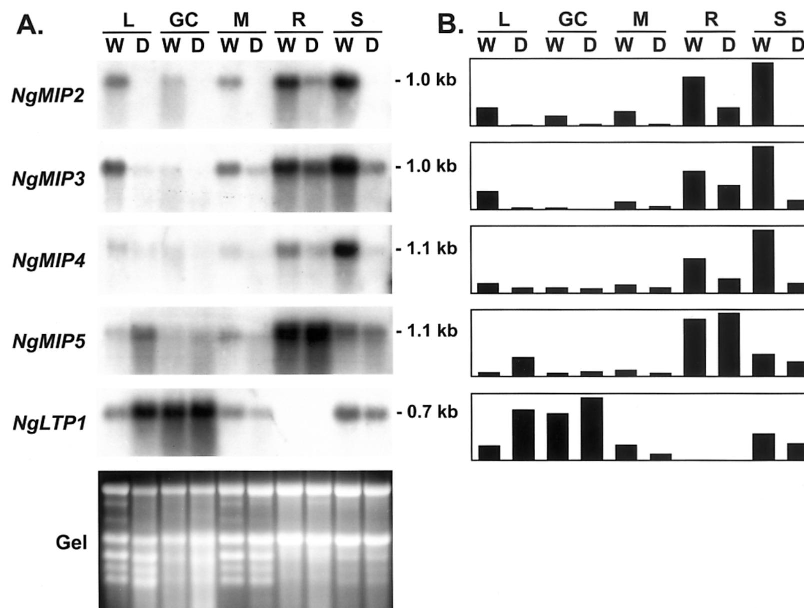

Fig. 4 Northern analysis of *NgMIP2*, *NgMIP3*, *NgMIP4*, *NgMIP5* and *NgLTP1* expression in well-watered and drought-stressed plants. (A) Blots contain 21 µg of *N. glauca* RNA isolated from either well-watered (W) or drought-stressed (D) leaves (L), guard cells (GC), mesophyll cells (M), roots (R) or stems (S). (B) Hybridization was quantified using a phosphorimager, and the relative signal intensities for each blot are shown in the bar graphs. A photo of a representative ethidium bromide-stained gel is shown to demonstrate approximately equal loading of intact samples. Sizes of bands calculated based on the migration of size standards are shown on the right.

Regulation of MIP gene expression under drought stress

In well-watered *N. glauca* plants, mRNA accumulation from *NgMIP2*, *NgMIP3* and *NgMIP4* was most abundant in stems, but was also relatively high in roots (Fig. 4). Transcripts from those three genes were detectable at lower levels in RNA from mesophyll cells and whole leaves of well-watered plants, while hybridization to guard cell RNA was far lower than to stem RNA and produced the lowest signal intensity of any well-watered samples tested (Fig. 4). Message from *NgMIP1* accumulated to highest levels in roots and stems in well-watered plants, with lower levels of expression in mesophyll cells and leaves, and lowest accumulation in guard cells (data not shown). Somewhat in contrast, mRNA levels from *NgMIP5* were 2.5× higher in roots than in stems of well-watered plants. As with *NgMIP4*, there were very low levels of *NgMIP5* transcript detected in RNA from whole leaves or mesophyll cells and lowest levels in RNA from guard cells of well-watered plants.

In plants that were experiencing acute drought stress, the message levels from *NgMIP2*, *NgMIP3* and *NgMIP4* dropped in all tissues surveyed, in some cases to very low or undetectable levels (Fig. 4). *NgMIP2* transcript was detectable only in RNA from roots of drought-stressed plants. For *NgMIP3* and *NgMIP4*, mRNA accumulation dropped approximately 40–50% in roots and more than 80% in stems of drought-stressed plants, while message levels in leaves, mesophyll cells and

guard cells also dropped dramatically (Fig. 4). In contrast, there was little difference in the accumulation of *NgMIP5* transcript in roots and only a slight decrease in stems of drought-stressed plants compared to well-watered plants. However, accumulation of *NgMIP5* mRNA was more than 4× higher in whole leaves of drought-stressed plants compared to well-watered plants (Fig. 4). To ensure that mRNA in the guard cell sample was intact, one of the blots was re-probed with a cDNA clone of *NgLTP1*, which encodes a drought-inducible, epidermis-specific lipid transfer protein (Smart et al. 2000). *NgLTP1* transcript levels were highest in guard cells and were induced 3.5× in whole leaves from drought-stressed plants (Fig. 4), indicating that these RNA preparations contained intact mRNA.

Discussion

Using a combination of degenerate oligonucleotide primers for PCR and moderate stringency library screening, we have isolated a total of five putative *TIP* and *PIP* genes from *N. glauca* guard cell cDNA and a guard cell cDNA library. The amino acid sequences deduced from these genes contain all of the features that are typical of aquaporins, including residues that may confer Hg sensitivity to the putative TIPs, NgMIP2 and NgMIP3, and those that possibly confer regulation by phosphorylation to the putative PIP, NgMIP4 (Johansson et al. 1998).

The deduced amino acid sequence of NgMIP3 is most similar (98.8% identity) to that of a putative TIP from *N. tabacum*, while the NgMIP2 and NgMIP4 deduced amino acid sequences are more similar to sequences from other plant genera, rather than sequences from *N. tabacum* or *N. excelsior*. *N. glauca* is a member of the Subgenus *Rustica*. *N. tabacum* is a member of the Subgenus *Tabacum* that resulted from amphiploidy involving progenitors of *Nicotiana sylvestris* (Subgenus *Petunioides*) and *Nicotiana otophora* (Subgenus *Tabacum*) (Goodspeed 1954). *N. excelsior* is a member of the Subgenus *Petunioides* (Goodspeed 1954), and thus its *MIP* genes should be more closely related to genes in *N. tabacum* than those from *N. glauca*. There are no *MIP* gene sequences that have been reported from other species in the Subgenus *Rustica*. NgMIP2 is closest in sequence similarity to a putative TIP isolated from cotton fibers, while NgMIP4 is closest to a putative PIP isolated from pulvini of *S. saman*. These similarities are intriguing, since cotton fibers differentiate from the epidermis and pulvinar motor cells undergo rapid volume changes similar to guard cells.

The MIP proteins are encoded by a multigene family in *N. glauca*, as they are in other plants. Studies of the aquaporin gene family are most complete for *A. thaliana* (Weig et al. 1997) and *M. crystallinum* (Tyerman et al. 1999, Kirch et al. 2000). Characterization of the Arabidopsis genome provides evidence for three subfamilies of genes, those encoding TIPs, PIPs and NLM1 (Weig et al. 1997). In *M. crystallinum*, those three subfamilies can be further divided into a total of six subgroups (Tyerman et al. 1999). Phylogenetic analysis of the genes from *N. glauca* indicates that *NgMIP2* and *NgMIP3* are members of group 2a, which includes Arabidopsis δ-*TIP* and γ-*TIP*, while *NgMIP4* is a representative of group 1b, which includes Arabidopsis *PIP2a*, *PIP2b*, *PIP3* and *RD28* (Fig. 2). PCR-based cloning resulted in the isolation of partial-length clones of genes, *NgMIP1* and *NgMIP5*, that are most similar to *NgMIP2* and *NgMIP4*, respectively. Furthermore, weak cross-hybridization on some Southern blots suggests that there are additional homologs of *NgMIP3* as well (Fig. 3). Thus, it is highly unlikely that we have isolated clones representing each of the members of the *MIP* gene family in *N. glauca*, although our approaches should have been effective in isolating the most abundantly expressed genes in guard cells.

The cDNA clones representing *NgMIP1* to *NgMIP5* were all isolated from a library made from a highly enriched preparation of guard cells (Smart et al. 2000). However, these genes are not expressed in a guard cell-specific manner, since they appear to be most abundantly expressed in stems, roots and also in mesophyll cells. Two aquaporins, SunTIP7 and SunTIP20, which were shown to be expressed in guard cells of sunflower, are also expressed in roots and peduncles, with SunTIP7 showing relatively high expression in stems and hypocotyls (Sarda et al. 1997, Sarda et al. 1999). Likewise, the Arabidopsis *PIP1b* gene was shown, by analysis of promoter-GUS fusion transgenic plants, to be expressed not only in guard cells, but also in roots and vasculature (Kaldenhoff et al. 1995). Although it must be considered that no aquaporin gene family has been fully characterized, the current body of evidence must be interpreted to say that there do not appear to be guard cell-specific aquaporin isoforms. The levels of *MIP* gene expression in *N. glauca* guard cell RNA were the lowest of any tissue analyzed. These results suggest that aquaporins play a more critical functional role in stems and roots, than in guard cells. Since the levels of expression in guard cells are so low in well-watered plants, it is not likely that down-regulation under drought stress would have a significant impact on water permeability of guard cell membranes or on stomatal opening or closure. In *N. glauca*, it is clear that there is overlapping expression of MIP genes within guard cells. There is also overlapping expression of multiple genes encoding phosphoenolpyruvate carboxylase in *N. glauca* guard cells (L.B. Smart, unpublished results) and of genes for the plasma membrane H^+-ATPase in guard cells of *Vicia faba* (Hentzen et al. 1996). These types of molecular data are important complements to biophysical and biochemical characterization of stomatal physiology and indicate that membrane transport or enzymatic activity in guard cells may represent the combined activities of multiple expressed isoforms.

The activity of aquaporins in a membrane can increase its water permeability dramatically (Wayne and Tazawa 1990). There are currently two opposite descriptions of the role of aquaporins in response to dehydration stress. The first is based on evidence that expression of some aquaporins is induced

under dehydration stress (Guerrero et al. 1990, Yamaguchi-Shinozaki et al. 1992, Fray et al. 1994, Yamada et al. 1997), which is predicted to result in greater membrane water permeability and facilitated water transport. The opposite seems to be true when aquaporin activity is down-regulated under dehydration stress, which should result in decreased membrane water permeability and may allow cellular water conservation (Yamada et al. 1995, Johansson et al. 1998). This role of aquaporins was described based on data that the spinach leaf PIP, PM28A, is preferentially phosphorylated under high water potential conditions, and that the activity of this aquaporin in *Xenopus* oocytes is dependent upon phosphorylation (Johansson et al. 1998). In the case of spinach, the accumulation of *PM28A* mRNA and protein are constitutively high and not affected by drought stress (Johansson et al. 1996).

In *N. glauca*, there appears to be coordinated expression of the putative *N. glauca TIP*s *NgMIP2* and *NgMIP3* and the putative *PIP NgMIP4* whose RNA levels dropped dramatically under drought stress. Although transcriptional regulation is a longer-term response than protein phosphorylation, one would predict that it is accomplishing a similar end result of decreasing the water permeability of the membranes under drought stress. Down-regulation of *MIP* genes was also observed in stressed leaves of ice plant (*M. crystallinum*) (Yamada et al. 1995). In sunflower, *SunTIP18* mRNA levels declined after 24 h of dehydration stress, while levels of *SunTIP7* mRNA increased and those of *SunTIP20*, *SunRb7* and *SunγTIP* remained approximately the same (Sarda et al. 1999). *N. glauca* is a species that is native to the western slopes of the Andes in Argentina and is well-adapted to arid conditions (Goodspeed 1954). The coordinated down-regulation of aquaporins in this plant may represent a mechanism that has evolved to allow greater cellular water conservation and improved drought tolerance.

We have learned a great deal about plant response to water deficit by studying the regulation of genes induced by drought and/or ABA (Bray 1997). However, there are relatively few examples of genes whose expression in vegetative tissue is down-regulated by water deficit. Genes that are down-regulated by water deficit stress include some that encode cell wall structural proteins; such as proline-, threonine-, glycine-rich protein (Yu et al. 1996, Harrak et al. 1999) and glycine-rich protein (de Oliveira et al. 1990, Keller and Baumgartner 1991). Also, a Class I chitinase-like gene from loblolly pine was shown to be down-regulated by water stress (Chang et al. 1996). It is possible that the decrease in *N. glauca MIP* gene mRNA upon drought stress occurs either by transcriptional down-regulation or by altered message stability. Future studies of the *N. glauca* MIP genes will be focused on characterizing the mechanisms of coordinated down-regulation under water deficit stress.

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