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Water-selective and multifunctional aquaporins from *Lotus japonicus* nodules

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Abstract. By using reverse transcriptase-polymerase chain reaction, two cDNAs were isolated that encode major intrinsic membrane proteins (MIPs) that are expressed in nitrogen-fixing root nodules of *Lotus japonicus*. *Lotus* intrinsic membrane protein 1 (LIMP 1) is expressed at high levels in both nodule and root tissues and shows highest sequence similarity to members of the tonoplast intrinsic protein (TIP) subfamily of plant MIPs. Functional analysis of LIMP 1 by expression in *Xenopus laevis* oocytes show that it is a water-specific aquaporin. In contrast, LIMP 2 shows the highest sequence similarity to soybean nodulin 26 (67.8% amino acid sequence identity). LIMP 2 is also a nodulin, showing expression only in mature nitrogen fixing nodules of *L. japonicus*. LIMP 2 is a multifunctional aquaglyceroporin, and displays the ability to flux both water as well as glycerol upon expression in *Xenopus* oocytes. Additionally, the carboxyl terminal region of LIMP 2 has a conserved phosphorylation motif that is phosphorylated by a calmodulin-like domain protein kinase. Overall, the data show that *L. japonicus* nodules contain two structurally and functionally distinct MIP proteins: one (LIMP 2) which appears to be the nodulin 26 ortholog of *L. japonicus* and another (LIMP 1) which appears to be a member of the TIP subfamily.

Key words: Aquaporin – Calcium-dependent protein kinase – *Lotus* (aquaporin) – Major intrinsic proteins – Nitrogen-fixing symbiosis – Nodulin 26

Introduction

Under limiting soil nitrogen conditions, legumes are infected by free-living rhizobia bacteria which trigger a developmental program that culminates in the formation of nitrogen-fixing root nodules. The bacteria become encapsulated within a specialized organelle, the symbiosome, within the infected host cells in the cortex of the nodule. The symbiosome membrane is host-derived and mediates the exchange of metabolites (efflux of fixed ammonium and the uptake of dicarboxylates as an energy source) between the host and symbiont (reviewed by Udvardi and Day 1997).

During the formation of the symbiosome, several host-encoded, nodule-specific proteins (nodulins) are targeted to the symbiosome membrane. Among these is the integral membrane protein nodulin 26 (Fortin et al. 1987), which constitutes 10–15% of the total symbiosome membrane protein (Weaver et al. 1991; Rivers et al. 1997). Nodulin 26 is a member of the major intrinsic protein (MIP) superfamily, an ancient family of membrane proteins (reviewed in Reizer et al. 1993; Froger et al. 1998). Functionally, MIPs mediate the flux of water (aquaporins), solutes such as glycerol (glyceroporins) or both (multifunctional aquaglyceroporins) (reviewed in Agre et al. 1998). In previous work we showed that soybean nodulin 26 is an aquaglyceroporin that mediates the high permeability of the symbiosome membrane to water and uncharged solutes (Rivers et al. 1997; Dean et al. 1999).

The control of water and solute homeostasis in plants is critical to a variety of physiological processes including cell volume regulation, transcellular water flow in response to changing water potential, the maintenance of cell turgor, and adaptation to osmotic stresses, and it has been proposed that MIP proteins play a role in the control of these processes (reviewed in Tyerman et al. 1999). The finding of a multifunctional aquaglyeroporin that is found solely on the symbiosome membrane argues for a specialized, symbiotic role. However, details of the symbiotic function of nodulin 26 have remained elusive. To address further the biological role of nodulin

Abbreviations: At NLM-1 = nodulin 26-like MIP of *Arabidopsis thaliana*; CDPK = calmodulin-like domain protein kinase; GlpF = glycerol facilitator of *Escherichia coli*; LIMP1 = *Lotus* intrinsic membrane protein 1; LIMP2 = *Lotus* intrinsic membrane protein 2; MIP = major intrinsic protein; PCR = polymerase chain reaction; PIP = plasma membrane intrinsic protein; RT = reverse transcriptase; SPCP = soybean putative channel protein; TIP = tonoplast intrinsic protein

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26, the ability to exploit a genetically tractable system would be advantageous. For legumes, such as soybean, that form determinant nodules, *Lotus japonicus* has emerged as a model organism (Handberg and Stougaard 1992). In the present study, we have identified and characterized two MIPs expressed in *L. japonicus* nodules, one of which appears to be the nodulin 26 ortholog, and another which appears to be a member of the tonoplast intrinsic protein (TIP) familiy of plant aquaporins.

Materials and methods

Plant growth conditions. *Lotus japonicus* B-129-56 'Gifu' seeds were scarified and planted in a 1:1 mix of sand and vermiculite and were grown under greenhouse conditions. On the day of planting, seedlings were watered with a 3-d-old culture of *Mesorhizobium loti* NZP 2235 (Stiller et al. 1997), diluted 1:1 with Herridges nutrient solution (K_2HPO_4, 22 mg/L; KH_2PO_4, 17 mg/L; KCl, 19 mg/L; $MgSO_4{\cdot}7H_2O$, 250 mg/L; $CaCl_2{\cdot}2H_2O$, 37 mg/L; ferric monosodium EDTA, 9 mg/L; H_3BO_3, 0.71 mg/ml; $MnCl_2{\cdot}4H_2O$, 0.45 mg/L; $ZnCl_2$, 0.03 mg/L; $CuCl_2{\cdot}2H_2O$, 0.01 mg/L; $NaMoO_4{\cdot}2H_2O$, 0.005 mg/L). The plants were re-inoculated with *M. loti* 7 d later, and were watered on alternative weeks with water or Herridges solution. Non-nodulated *Lotus japonicus* were grown as described above in the absence of *M. loti* and with Herridges solution containing 5 mM KNO_3.

Isolation of nodule MIP cDNAs. Total RNA was extracted from 9-week-old *Lotus japonicus* nodules by a guanidinium thiocyanate procedure (Sambrook et al. 1989) and was purified by differential LiCl precipitation (Ausubel et al. 1987). Purified total RNA (5 µg) was used for first-strand cDNA synthesis by using reverse transcriptase (RT), an oligo dT primer and a 3′RACE system (Gibco BRL, Gaithersburg, Md., USA). The cDNAs corresponding to MIP proteins were amplified by touchdown polymerase chain reaction (PCR) with degenerate primers (5′-GGTGG[CT]CA[CT][TG]T[CT]AA[CT]CC[AT]GCTGT[CG]AC-3′ and 5′-CTTCT[AG]GC[CT]GG[AG]TTCAT[TG]GATGC[AT]-CC-3′) based on the highly conserved NPA motif sequences found in MIP proteins (Reizer et al. 1993; Froger et al. 1998).

Amplification was done with a Perkin Elmer GeneAmp PCR system 2400 with the following cycling conditions: denaturation 94 °C, 1 min; annealing 30 s; extension 72 °C 2 min. The annealing temperature was decreased in two-degree steps every third cycle from 48 °C to 40 °C, followed by three cycles at 39 °C, and a final fifteen cycles with an annealing temperature of 48 °C. An aliquot of the final product was re-amplified by PCR, and was cloned using the pCR 2.1 TA cloning system (Invitrogen, San Diego, Calif., USA). Clones encoding two MIP analogs, LIMP 1 and LIMP 2, were identified and pursued further.

A cDNA library prepared from mature *Lotus japonicus* nodules and cloned into λ Uni-ZAP XR was graciously supplied by Dr. Jens Stougaard, Aarhus, Denmark. The library was screened by replicate plating of plaques onto nitrocellulose filters by using the manufacturer's protocol (Custom Lambda Phage Libraries; Stratagene, La Jolla, Calif., USA). Radiolabeled probes were generated by nick translation (Nick Translation Kit; Promega, Madison, Wis., USA) of cloned PCR fragments corresponding to LIMP 1 or LIMP 2. These were hybridized with nitrocellulose membranes in 50% (v/v) formamide, 5 × SSPE (1 × SSPE = 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7), 5 × Denhardt's solution (1 × Denhardt's = 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 0.1% (w/v) SDS, 100 µg/ml denatured salmon sperm DNA, and 10^6 cpm probe/ml at 42 °C for 16 h. The filters were washed in 2 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M Na_3-citrate, pH 7), 0.1% (w/v) SDS and then with 1 × SSC, 0.1% (w/v) SDS (Sambrook et al. 1989). Positive plaques, identified by autoradiography, were selected and re-screened until plaque purified. Plasmids containing the cDNAs were excised as outlined in the ZAP-cDNA SYNTHESIS KIT manual (Stratagene). Automated DNA sequencing was performed on a Perkin Elmer Applied Biosystems 373 DNA sequencer at the University of Tennessee Molecular Biology Research Facility (Knoxville, Tenn., USA). Reactions were prepared with a Prism Dye Terminator Cycle sequencing kit (Perkin Elmer Applied Biosystems, Foster City, Calif., USA).

Northern blot analysis. Total RNA samples from 9-week-old *Lotus japonicus* plants were resolved by electrophoresis on 1.2% (w/v) agarose gels in the presence of 5.6% (w/v) formaldehyde (Sambrook et al. 1989). The RNA was blotted onto Zeta-Probe (Biorad, Hercules, Calif., USA) membranes under vacuum. The membranes were washed in 2 × SSC, dried overnight, and were crosslinked with a UV Stratalinker 2400 (Stratagene). Membranes were prehybridized with 0.5 M $NaPO_4$ (pH 7.2), 7% (w/v) SDS for 10 min at 65 °C, and were hybridized in the same solution containing 10^6 cpm/ml of various nick-translated radiolabeled probes. The membranes were washed twice at 65 °C in 1 mM EDTA, 40 mM $NaPO_4$ (pH 7.2), 5% (w/v) SDS for 30 min and were exposed to X-ray film at −80 °C. Under these hybridization conditions there was no cross-hybridization observed between LIMP 1 and LIMP 2 transcripts.

Water and glycerol permeability measurements in Xenopus laevis oocytes. Polymerase chain reaction with the *Pfu* polymerase and the following primers was done to engineer *Bgl* II sites flanking the open reading frames of LIMP 1 and LIMP 2. For LIMP 2: forward, GAAGATCTTGTTTTAGCTAGTGAGTG; reverse, GAAGATCTAACCACTAGGCGTCTAGGC; For LIMP 1: forward, GAAGATCTCGTTTTCCTACAGTCGCCG; reverse, GAAGATCTCATTTTCACTTTCACTTCCC.

The LIMP 1 and LIMP 2 PCR products were gel-purified, digested with *Bgl* II, and cloned into the *Bgl* II site of pXBG-ev1 plasmid downstream of the T3 RNA polymerase promoter as previously described (Rivers et al. 1997). The cRNA was synthesized by in-vitro transcription and was expressed in *Xenopus laevis* oocytes as previously described (Rivers et al. 1997; Dean et al. 1999). The osmotic water permeability of oocytes was measured by assaying the initial rates of oocyte swelling upon rapid dilution from iso-osmotic Ringer's solution (96 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$, 0.6 mM $CaCl_2$, 5 mM Hepes-NaOH, pH 7.6, 202 mOsm/kg) to hypoosmotic dilute Ringer's solution (59 mOsm/kg). Swelling was measured by video microscopy on a Nikon Alphaphot YS microscope equipped with a Pro-Series High Performance CCD Camera. Images were captured and digitized by the Image-Pro Plus software (Media Cybernetics Silver Spring, Md., USA). The cross-sectional area of the oocyte was determined, and used to calculate the oocyte volume (Eq. 1).

$$\left(\frac{A}{A_0}\right)^{\frac{3}{2}} = \frac{V}{V_0} \tag{1}$$

where A is the cross-sectional area at a given time; A_0 is the initial cross-sectional area; V is the oocyte volume at the given time, V_0 is the initial oocyte volume. The change in oocyte volume (V/V_0) was measured over time to determine an initial swelling rate, $(dV/V_0)/dt$, from which the osmotic water permeability P_f was calculated (Eq. 2).

$$P_f = \frac{(V_0/S_0)(dV/V_0)/dt}{(S_{real}/S_{sphere})V_w(osm_{in} - osm_{out})} \tag{2}$$

where, S_0 is the initial oocyte surface area, osm_{in} is the osmolarity in the oocyte, osm_{out} is the osmolarity of the medium, V_w is the partial molar volume of water, S_{real} is the actual area of the oolemma and S_{sphere} is the area calculated by assuming a sphere. S_{real}/S_{sphere} is taken as 9 for all measurements (Rivers et al. 1997). The permeability of *Xenopus* oocytes to [^{3}H]glycerol was measured as described in Dean et al. (1999).

Protein kinase analysis. Assay of calmodulin-like domain protein kinase (CDPK) was done by a phosphocellulose filter paper method (Weaver et al. 1991). Assays were done in 25 mM Mops-NaOH (pH 7.0), 10 mM magnesium acetate, 7.5 mM β-mercaptoethanol, 0.1 mM [γ^{32}P]ATP (980 dpm/pmol), and various amounts of a synthetic peptide substrate (CI-14; Bio-Synthesis, Lewisville, Tex., USA). CI-14 contains the 13 residues at the carboxyl terminus of LIMP 2 along with an amino-terminal cysteine to allow quantitation with Ellman's reagent. Assays were initiated by the addition of a recombinant CDPK (KJM23-6H2) (Harper et al. 1994).

Results

Isolation of LIMP 1 and LIMP 2 cDNA sequences from Lotus japonicus. To identify MIP proteins expressed in the nodules of *L. japonicus*, RT-PCR was done using degenerate primers based on the highly conserved NPA motifs of MIPs (Reizer et al. 1993; Froger et al. 1998). The RT-PCR of total RNA from nodules of 9-week-old *Lotus japonicus* plants showed a major 370-bp product which was cloned into pCR 2.1. Based on sequence analysis of the cloned products, two MIP proteins, designated LIMP 1 and LIMP 2 (Lotus Intrinsic Membrane Protein 1 and 2), were identified.

The cloned PCR products were used to isolate two full-length cDNA clones for LIMP 1 and LIMP 2 (Fig. 1). The 1211-bp LIMP 2 cDNA contains an open reading frame that codes for a protein of 270 amino acid residues (Fig. 1A), as well as 91 bp of untranslated 5′ sequence (including an in-frame stop codon upstream of the ATG start site) and 310 bp of untranslated 3′ sequence. The 1162-bp LIMP 1 cDNA (Fig. 1B) contains an open reading frame that codes for a protein of 251 amino acid residues, as well as 59 bp of untranslated 5′ sequence (including an in-frame stop codon upstream of the ATG start site) and 350 bp of untranslated 3′ sequence.

Analysis of the deduced amino acid sequences showed that LIMP 1 and 2 share only 23.5% amino acid identity, indicating the two proteins are highly divergent. The LIMP 2 protein shared the highest sequence similarity (67.8% identity) with soybean nodulin 26, whereas the LIMP 1 open reading frame shared a high identity (83.9%) with the soybean putative channel protein SPCP 1 (Miao and Verma 1993). Both proteins contain all the hallmarks of the MIP family (Reizer et al. 1993) including the highly conserved NPA regions and six predicted transmembrane α-helical domains (Fig. 2). The overall positioning and magnitude of these domains between LIMP 2 and nodulin 26 (Fig. 2A), and LIMP 1 and SPCP 1 (Fig. 2B) are remarkably similar, further underscoring the high degree of structural relatedness between each pair of proteins.

Organ-specific expression of LIMP 1 and LIMP 2 mRNA. The representation of both LIMP 1 and LIMP 2 in the cDNA library suggests that these proteins are expressed in nodules, but it remains unclear whether they are nodule-specific proteins (i.e. nodulins). Northern blot analysis with a LIMP 2-specific probe showed the presence of a hybridizing mRNA signal of the expected size in total RNA isolated from nodules of *L. japonicus* but no detectable message in either roots or leaves and stems (Fig. 3A). In contrast, LIMP 1 showed high expression in roots and nodules, but its expression was markedly reduced in leaves and stems (Fig. 3B). Overall, the data suggest that LIMP 2 appears to be a *Lotus japonicus* nodulin whereas LIMP 1 is expressed principally in roots and nodules.

Phosphorylation of CI-14 by CDPK. Examination of the carboxyl terminal region of the LIMP 2 sequence revealed a consensus hydrophobic-X-basic-X-X-ser phosphorylation motif (ITKNVS, residues 260–265, Fig. 1A) for CDPKs (Bachmann et al. 1996). To test whether LIMP 2 is phosphorylated by CDPK, a synthetic peptide, CI-14, was synthesized that contains the terminal 14 amino acids of LIMP 2. CI-14 was readily phosphorylated by CDPK (Fig. 4) with an apparent K_m of 178 μM.

Functional transport properties of LIMP 1 and LIMP 2 in Xenopus laevis. *Xenopus laevis* oocytes injected with capped LIMP 1 and LIMP 2 cRNA showed a 3- to 4-fold higher rate of swelling than negative control oocytes upon immersion in hypoosmotic medium (Fig. 5A). Based on the swelling rate and the osmotic gradient, we calculated the osmotic water permeability (P_f) for oocytes injected with LIMP 2 (3.2×10^{-4} cm/s) and LIMP 1 (4×10^{-4} cm/s) (Fig. 5B). Both values are similar to that previously found for soybean nodulin 26 (3.5×10^{-4} cm/s) (Rivers et al. 1997).

Nodulin 26- and LIMP 2-injected oocytes showed a high rate of [^{3}H]glycerol uptake (Fig. 5C), which was virtually identical to that of oocytes injected with the glycerol facilitator of *Escherichia coli*, GlpF (data not shown). In contrast, the LIMP 1 protein did not show any significant flux of glycerol over water-injected control oocytes (Fig. 5C). Based on these results we conclude that LIMP 2 and nodulin 26 form multifunctional aquaglyceroporins that transport both water and uncharged solutes such as glycerol, whereas LIMP 1 forms a water-selective aquaporin.

Discussion

Analysis of multiple protein sequences of plant MIPs (Weig et al. 1997) shows that they can be divided into three subgroups: the tonoplast intrinsic proteins (TIPs), plasma-membrane intrinsic proteins (PIPs), and a third class composed of soybean nodulin 26 and *Arabidopsis thaliana* nodulin 26-like MIP (At NLM-1; Weig et al. 1997). In the present study, RT-PCR was used to identify two new MIP family members, LIMP 1 and LIMP 2 from *Lotus japonicus*. Comparisons with other MIP members show that LIMP 1 and LIMP 2 cluster into two distinct MIP subclasses (Fig. 6). LIMP 1 is a member of the TIP subclass with highest similarity to SPCP 1 (83.9% identity) (Miao and Verma 1993) and *Arabidopsis thaliana* γTIP (78.1% identity) (Höfte et al.

A

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GAATTCGGCACGAGGTGGTCCATCTCTTTTCCCATCTCAAAGGAAACAAAACTCCCCTGTCCCTTTATTTCCTTGTTTTA
GCTAGTGAGTGATGGGTGAGAGTTCCGCACCCAATGGACTGAATGGAGCTCATGAGGTAGTTGTAAATGTAAACAAGGAT
            M  G  E  S  S  A  P  N  G  L  N  G  A  H  E  V  V  V  N  V  N  K  D
GCCTCTAAGACAATTGAAGTCTCAGACACCAACTTCACTGTTTCTTTCTTGCAGAAGGTAATAGCTGAGTTGGTGGGTAC
A  S  K  T  I  E  V  S  D  T  N  F  T  V  S  F  L  Q  K  V  I  A  E  L  V  G  T
TTATTTTTTTATATTTGCTGGTTGTGCTTCCATTGTGGTGAACAAGAACAATGACAATGTGGTCACACTTCCTGGTATTG
   Y  F  F  I  F  A  G  C  A  S  I  V  V  N  K  N  N  D  N  V  V  T  L  P  G  I
CACTTGTCTGGGGACTGGCTGTGATGGTGCTGGTTTACTCTCTTGGTCACATTTCTGGTGCCCATTTCAATCCTGCTGCC
A  L  V  W  G  L  A  V  M  V  L  V  Y  S  L  G  H  I  S  G  A  H  F  N  P  A  A
ACCATTGCATTTGCCTCCACCAAAAGGTTTCCCTGGAAGCAGGTACCAGCTTATGTATCAGCCCAGGTCCTTGGATCCAC
   T  I  A  F  A  S  T  K  R  F  P  W  K  Q  V  P  A  Y  V  S  A  Q  V  L  G  S  T
ACTTGCAAGTGGAACTCTTAGACTAATATTCAGTGGCAAGCATAACCAATTTGCAGGAGCACTCCCAACTGGGTCTAATC
   L  A  S  G  T  L  R  L  I  F  S  G  K  H  N  Q  F  A  G  A  L  P  T  G  S  N
TGCAAGCTTTTGTGATTGAATTCATAATCACTTTTTTCCTTATCTTTATCTTATTTGGGGTTGCCACTGATGATCGAGCG
L  Q  A  F  V  I  E  F  I  I  T  F  F  L  I  F  I  L  F  G  V  A  T  D  D  R  A
ATTGGTGAGGTTGCTGGGATTGTGGTGGGATCTACAGTGCTTTTGAATGTGTTGTTTGCAGGGCCAATAACTGGAGCATC
   I  G  E  V  A  G  I  V  V  G  S  T  V  L  L  N  V  I  F  A  G  P  I  T  G  A  S
AATGAACCCAGCAAGAAGCATAGGGTCTGCTTTTGTACACAATGAGTACAGAGGAATATGGATATACTTGCTATCTCCAA
   M  N  P  A  R  S  I  G  S  A  F  V  H  N  E  Y  R  G  I  W  I  Y  L  S  P
CTCTGGGGGCAGTGGCTGGTGCATGGGTCTATAACATCGTTCGCTACACAGACAAGCCATTGCGTGAGATCACTAAAAAT
T  L  G  A  V  A  G  A  W  V  Y  N  I  V  R  Y  T  D  K  P  L  R  E  I  T  K  N
GTCTCTTTTCTCAAAGGAATATGAGAGTCCTAAAAGCCTAGACGCCTAGTGGTTATGGATCACTATGCCAAGAGAAACAT
V  S  F  L  K  G  L
CAAACTTCTCTAAGATTGGAATAATTTTATCCATCCTTAAGATGCTAGTAGTTATTAGTCGCATTTCTACTTTATGGTTG
TTAGGAGAATGTTGAGTTGCCAATATTCTTTTGGTGGCTTTGTTATTCTGTTTGTTATTTTGCATATGTTATTTCCATTT
AGTTACTATATTGTAAGCCTTTATAAAGTGATGAAAGTAATATTGGATAAATGCATTGAGCCAATCTTAAAATAAAAAAA
AAAAAAAAAAA 1211
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B

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GAATTCGGCACGAGCACAGTGAGTGTTGATCATCGTTTTCCTACAGTCGCCGGAAGAAGATGCCGATCCGCAACATCGCC
                                                  M  P  I  R  N  I  A
ATCGGTGCCCCTCAGGAGGCAACCCACCCAGACACCTTGAGGTCAGCCTTGGCTGAGTTCATCTCCACCTTCATCTTCGT
I  G  A  P  Q  E  A  T  H  P  D  T  L  R  S  A  L  A  E  F  I  S  T  F  I  F  V
CTTCGCCGGCTCCGGTTCCGGAATCGCCTACAACAAACTCACCGACGACGGCGCCGCCACCCCCGCCGGCCTCATCTCCG
   F  A  G  S  G  S  G  I  A  Y  N  K  L  T  D  D  G  A  A  T  P  A  G  L  I  S
CCTCCATCGCCCACGCATTCGCCCTCTTCGTCGCCGTCTCCATCAGCGCCAACATCTCCGGCGGCCACGTCAACCCCGCC
A  S  I  A  H  A  F  A  L  F  V  A  V  S  I  S  A  N  I  S  G  G  H  V  N  P  A
GTCACCTTCGGCCTCTTCGTCGGCGGTAACATCTCCTTCCTCCGCGGTGTCATCTACATCATCGCTCAGCTTCTCGGATC
V  T  F  G  L  F  V  G  G  N  I  S  F  L  R  G  V  I  Y  I  I  A  Q  L  L  G  S
CATCGTCGCCTCCTTGCTCCTCGCCTTCGTCACCGGACTCGCTGTACCTGCTTTCGGTCTTTCAGCTGGAGTTGGAGTTG
   I  V  A  S  L  L  L  A  F  V  T  G  L  A  V  P  A  F  G  L  S  A  G  V  G  V
GGAACGCGTTGGTGTTGGAGATCGTGATGACCTTTGGATTGGTGTACACAGTTTACGCCACAGCCGTTGATCCCAAGAAG
G  N  A  L  V  L  E  I  V  M  T  F  G  L  V  Y  T  V  Y  A  T  A  V  D  P  K  K
GGTAGTTTGGGAACTATTGCACCTATTGCTATTGGTTTCATTGTTGGTGCTAACATTTTGTTGGGTGGGGCATTCGACGG
   G  S  L  G  T  I  A  P  I  A  I  G  F  I  V  G  A  N  I  L  L  G  G  A  F  D  G
AGCATCCATGAACCCCGCCGTGTCATTCGGACCAGCCGTCGTGAGCTGGAGCTGGTCTAACCACTGGATCTACTGGGTCG
   A  S  M  N  P  A  V  S  F  G  P  A  V  V  S  W  S  W  S  N  H  W  I  Y  W  V
GACCACTTGTTGGTGGTGGTATTGCTGGACTTATCTATGAGGTGGTTTTCATTGGCAGCCAAACCCATGAACAGCTTCCT
G  P  L  V  G  G  G  I  A  G  L  I  Y  E  V  V  F  I  G  S  Q  T  H  E  Q  L  P
ACTACTGACTACTAGAGGGAAGTGAAAGTGAAAATGAAAATTGTGTATTATTGAATTTCTCTGAATTTTTCCTTTGTGCA
T  T  D  Y
TCATGTTTCATTCCACTCTTGTCCTTTATTTTATCTTCAAAAATTGGGAGTTGTTTCTTTTGTGTTGAATGAATTGGTGG
CTGTTGTTGATCTGTGGTTCTATGGTTGTATGGTTGATGATCATGTAAGTTATGTTCCAAACAGCTCCACCATTGAAATT
GAATGTTAATTATTTGGGTCTGGTTGGATGTTTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 1162
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Fig. 1A,B. Nucleotide sequences for LIMP 2 and LIMP 1 cDNAs. **A** Nucleotide sequence for LIMP 2 cDNA showing a deduced open reading frame of 270 amino acid residues. **B** Nucleotide sequence for LIMP 1 cDNA showing a deduced full-length open reading frame of 251 amino acid residues

1992). In contrast, LIMP 2 is a member of the nodulin 26 subclass with high similarity to soybean nodulin 26 (Fortin et al. 1987) (67.8% identity) and At NLM-1 (Weig et al. 1997) (61.9% identity).

The clustering of LIMP 1 and LIMP 2 into these distinct plant MIP subclasses is also reflected by the differences in their functional characteristics. The MIP family members fall into two broad functional categories: aquaporins that selectively transport water across biological membranes, and aquaglyceroporins and glyceroporins that show the ability to transport small uncharged solutes (Agre et al. 1998). Similar to γTIP (Maurel et al. 1993), LIMP 1 forms a water-selective pore when injected into *Xenopus* oocytes. In contrast, LIMP 2, similar to soybean nodulin 26 (Rivers et al. 1997; Dean et al. 1999), shows the ability to flux water as well as glycerol.

A comparison of the soybean nodulin 26, At NLM-1, and LIMP 2 sequences reveals some interesting parallels with other glycerol transport MIPs. Based on multiple sequence alignments of various aquaporins and glyceroporins, Froger et al. (1998) proposed five ''discriminant'' residues (P1–P5) that distinguish water-selective MIP proteins from those that transport solutes. Inter-

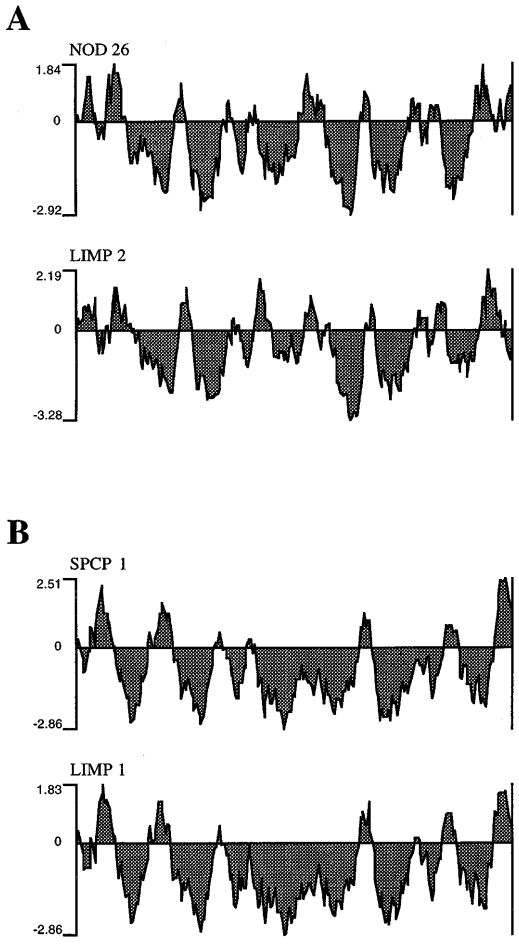

Fig. 2A,B. Hydropathy plots of LIMP 1 and LIMP 2. **A** LIMP 2 compared to soybean nodulin 26. **B** LIMP 1 compared to soybean SPCP1. Hydropathy plots were constructed using a sliding window of nine residues using the PROTEAN program of DNASTAR

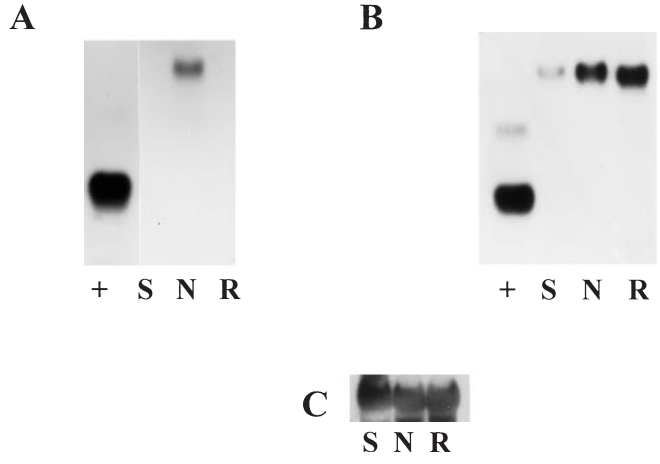

Fig. 3A–C. Northern blot of RNA from various *Lotus* tissues probed for the LIMP 2 or LIMP 1 transcript. Total RNA (10 μg/lanė) isolated from nodules (*N*), stem and leaves (*S*), and roots (*R*) of 9-week-old *Lotus japonicus* plants was analyzed by a Northern blot protocol as described in the *Materials and methods*. Blots were probed with the labeled cloned PCR fragments for LIMP 2 (**A**), LIMP 1 (**B**) or with a labeled 18S rRNA probe from rice (Zarembinski and Theologis 1993) as a loading control (**C**) shows PCR product for LIMP1 or LIMP2

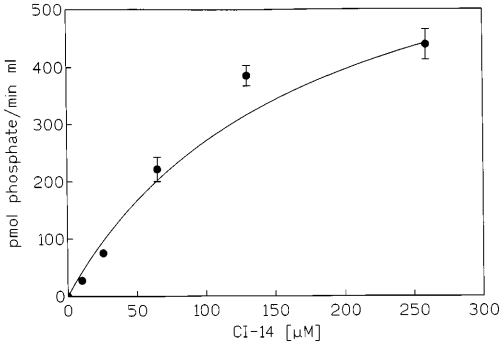

Fig. 4. The carboxyl terminal domain of LIMP 2 is phosphorylated by CDPK. The CI-14 peptide, containing the 13 carboxyl terminal residues of LIMP 2 (see Fig. 1A) was tested as a substrate for CDPK. Error bars depict SE

estingly, members of the nodulin 26 subfamily possess a sequence at these five positions that is a hybrid of two signature motifs (Table 1), with the P1 and P5 residues showing similarity to glycerol-transport MIPs whereas P2, P3, and P4 show similarity to aquaporins. In a recent study, Lagrée et al. (1999) showed that the residues at P4 and P5 within the sixth transmembrane segment are the key residues that determine glycerol selectivity in the glycerol facilitator GlpF. Whether the presence of a leucine for a tryptophan at position 5 is adequate to confer multifunctional transport on nodulin 26 and LIMP 2 remains to be addressed. All members of the nodulin 26 subfamily possess this motif, and it will be interesting to determine whether they also are aquaglyceroporins.

Several other genes encoding plant MIPs with these signature sequences have been identified in *Arabidopsis*, suggesting that the At-NLM/nodulin 26 family is more widespread than orginally described (Weig et al. 1997). Further, recent evidence shows that glycerol-transporting MIPs, such as the tobacco Nt-TIPa (Gerbeau et al. 1999) and NtAQP1 (Biela et al. 1999) proteins, exist in other non-legume species, indicating that aquaglyceroporins are found on other plant membranes besides the symbiosome membrane. However, LIMP 2 appears to represent the nodulin 26 ortholog of *L. japonicus* since it exhibits higher identity to soybean nodulin 26 than to any other MIP sequence, and shows nodule-specific expression, suggesting it is a bonafide nodulin. Further support comes from Western blot analysis of isolated *L. japonicus* symbiosome membranes with a LIMP 2-specfic antibody (directed against the CI-14 peptide) that shows that LIMP 2 is localized to this membrane (data not shown).

Another common feature of the nodulin 26 subfamily is the presence of a conserved CDPK phosphorylation motif within the hydrophilic carboxyl terminal region of the protein. Soybean nodulin 26 is the major substrate of a CDPK that is co-localized to the symbiosome membrane and which specifically phosphorylates the serine within this motif (Weaver et al. 1991; Weaver and Roberts 1992). Several animal and plant MIP proteins are phosphorylated by various protein kinases. Reports of the effects of phosphorylation on MIP transport

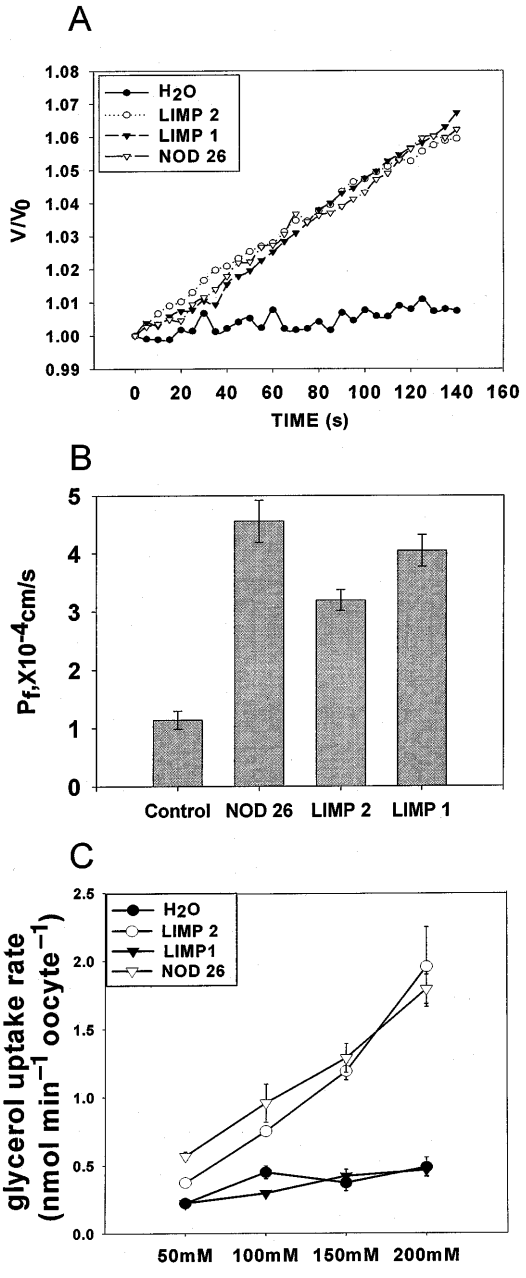

Fig. 5A–C. Water and glycerol permeabilities of LIMP 1 and LIMP 2 expressed in *Xenopus* oocytes. **A** Initial swelling rates of *Xenopus laevis* oocytes injected with 46 ng of the indicated cRNA or nuclease-free water (control). The rate of oocyte swelling upon immersion in hypoosmotic medium is plotted as V/V_0 versus time, where V is the volume at a given time point and V_0 is initial volume. **B** Osmotic water permeability (P_f) of LIMP 1- and LIMP 2-injected oocytes. The P_f values were calculated from the initial rate of oocyte swelling. Error bars depict SE ($n = 8$). **C** Glycerol uptake rate for *Xenopus* oocytes. [^{3}H]glycerol uptake of oocytes incubated in various glycerol concentrations from 50 to 200 mM. Error bars depict SE ($n = 12$)

activity are varied, including the stimulation of activity (Maurel et al. 1995; Johansson et al. 1998), the inhibition of activity (Han et al. 1998), and modulation of membrane targeting (Fushimi et al. 1997). Phosphorylation of nodulin 26 by CDPK affects its gating properties in planar lipid bilayers (Lee et al. 1995), but its role in control of water and solute transport in vivo

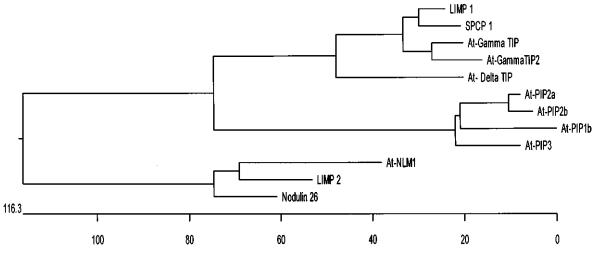

Fig. 6. Phylogenetic alignment of multiple plant MIP proteins showing the position of LIMP 1 and LIMP 2. Cladogram phylogenetic alignments were constructed using the DNASTAR software. Branch distances correspond to sequence divergence. Bottom scale units indicate the number of substitution events

Table 1. Comparison of five aquaporin/glyceroporin "discriminant" residues in LIMP 1 and LIMP 2

Residue[a]	P1	P2	P3	P4	P5
Aquaporin consensus	T/A	S	A	Y/F	W
Glycerol consensus	Y/F	D	K/R	P/A	I/L
Soybean nodulin 26	F	S	A	Y	L
L. japonicus LIMP 2	F	S	A	Y	L
At NLM-1	F	S	A	Y	L
L. japonicus LIMP 1	T	S	A	Y	W
SPCP 1	T	T	A	Y	W

[a]P1–P5 correspond to the discriminant residues discussed by Froger et al. (1998). P1 is found within loop C, P2 and P3 are found in the loop D containing the second NPA motif, and P4 and P5 are found in the sixth transmembrane region. Consensus sequences for aquaporin or glycerol transport MIPs are based on Froger et al. (1998)

needs to be rigorously addressed. However, the conservation of this CDPK phosphorylation site among nodulin 26 subfamily members suggests that regulation by calcium-dependent phosphorylation may be another shared characteristic.

The function that LIMP 2/nodulin 26 plays in the nitrogen-fixing symbiosis remains a open question. Based on previous studies, this is the major protein component that mediates the high permeability of the symbiosome membrane to water and uncharged solutes (Rivers et al. 1997; Dean et al. 1999). Since the symbiosome is the major organelle of the infected cell, nodulin 26 may play an osmoregulatory function, mediating the rapid, reversible uptake and release of water from the symbiosome space to control cytosolic and infected cell volume homeostasis similar to the proposed role of vacuolar water channels (reviewed in Tyerman et al. 1999). In addition, the control of the osmolarity of the symbiosome space may be critical for the nitrogen-fixing bacteroid. The role of nodulin 26/LIMP 2 becomes even more complex when one considers the added function of solute transport. Based on genetic analyses, glycerol-transport MIPs have been proposed to mediate glycerol flux for metabolic (e.g. GlpF; Sweet et al. 1990) and osmoregulatory processes (e.g. Fps1, the glycerol uptake/efflux facilitator protein of *Saccharomyces cerevisiae*; Luyten et al. 1995). However, the biological significance of the solute-transport function of most

aquaglyceroporins, including nodulin 26, remains unknown. Recent evidence suggests that MIP proteins can also enhance the permeability of membranes to gases (Nakhoul et al. 1998), and the possibility that nodulin 26/LIMP 2 aids in the exchange of gases such as CO_2, O_2 and NH_3 across the symbiosome membrane needs to be addressed.

LIMP 1 represents a second major aquaporin expressed in *L. japonicus* nodules, and it is likely that it plays a role distinct from that of LIMP 2. Besides being a water-specific aquaporin, LIMP 1 shows a different expression pattern, with high expression in nodules as well as roots. In this regard, LIMP 1 is similar to SPCP 1 (Miao and Verma 1993) which may serve a similar function in soybean. A role for water transport, perhaps mediated by aquaporins, has been suggested in the regulation of the O_2-diffusion barrier in nodules (Denison and Kinraide 1995; Serraj et al. 1998). The control of pO_2 within the nodule cortex is critical for the regulation of the rate of nitrogen fixation and is modulated in response to numerous environmental conditions (Hunt and Layzell 1993). The cells of the inner cortex of nodules are proposed to constitute the O_2-diffusion barrier, and are postulated to regulate gas diffusion by reversible water flux resulting in changes in cell volume and shape that restrict gas flow through the intercellular spaces (Denison and Kinraide 1995). Recently, high levels of MIP in the inner cortical cells of soybean nodules have been reported (Serraj et al. 1998). Whether LIMP 1 shows a similar distribution and whether it plays an essential role in controlling gas diffusion within the nodule remains unknown.

The ability to genetically transform *L. japonicus* with sense and antisense constructs of both LIMP 1 and LIMP 2 is now feasible, and will allow more meaningful testing of these various hypotheses of the roles of these proteins in symbiotic nitrogen fixation.

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