Review

The structure, function and regulation of the nodulin 26-like intrinsic protein family of plant aquaglyceroporins

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Abstract

The nodulin 26-like intrinsic protein family is a group of highly conserved multifunctional major intrinsic proteins that are unique to plants, and which transport a variety of uncharged solutes ranging from water to ammonia to glycerol. Based on structure–function studies, the NIP family can be subdivided into two subgroups (I and II) based on the identity of the amino acids in the selectivity-determining filter (ar/R region) of the transport pore. Both subgroups appear to contain multifunctional transporters with low to no water permeability and the ability to flux multiple uncharged solutes of varying sizes depending upon the composition of the residues of the ar/R filter. NIPs are subject to posttranslational phosphorylation by calcium-dependent protein kinases. In the case of the family archetype, soybean nodulin 26, phosphorylation has been shown to stimulate its transport activity and to be regulated in response to developmental as well as environmental cues, including osmotic stresses. NIPs tend to be expressed at low levels in the plant compared to other MIPs, and several exhibit cell or tissue specific expression that is subject to spatial and temporal regulation during development.
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Contents

1. Introduction

Major Intrinsic Proteins (MIPs) are integral membrane proteins that mediate the bidirectional flux of water and some small solutes across cellular membranes. MIPs are found in nearly all organisms, but are especially prevalent in plants, with over 30 genes typically found in higher plant genomes [1–4]. Phylogenetically, these gene products can be further divided into four subfamilies: Plasma membrane Intrinsic Proteins (PIPs), Tonoplast Intrinsic Proteins (TIPs), Nodulin 26-like Intrinsic Proteins (NIPs), and Small basic Intrinsic Proteins (SIPs). Thus, plant genomes consistently have several fold more MIP genes than animals and microbes, likely reflecting multiple roles of these proteins in the complex water relations guiding plant

development and responses to environmental stresses [5–7]. In addition, the plant MIPs have highly divergent pore structures [8] and many are proposed to be multifunctional transporters of varied solutes besides water ranging from reactive oxygen species [9] to gases [10] to metabolic substrates [11–15].

The NIP subfamily represents one such group of multifunctional MIPs that are named for their sequence similarity to the archetype of the family, soybean nodulin 26. Structurally and functionally, the NIP family constitutes a unique group of major intrinsic proteins that are permeable to a wide but defined range of small solutes [11–13,16–21]. Compared to the more widely expressed TIP and PIP genes, NIP genes are generally expressed at low levels [22]. In addition, they are often expressed in specialized cells and organs (such as nitrogen-fixing root nodules, discussed below) suggesting that NIP transport activities may be prevalent in a more defined set of cells in the plant. In this review, we discuss the current knowledge of the structure, function and regulation of the NIP family as well as their potential biological function in planta.

2. Soybean nodulin 26: a symbiosis-specific MIP

Free living diazotrophic soil bacteria of the Rhizobiaceae family colonize the roots of leguminous plants under limiting conditions of soil nitrogen to form a novel organ termed the nodule. The bacteria enter a symbiotic relationship with the plant in which the plant provides the bacteria with reduced carbon to support the energetic cost of the fixation of atmospheric dinitrogen to ammonia (reviewed in [23,24]). The infection of the plant by rhizobia bacteria is a host-specific process that is initiated by the secretion of bacterial signals called nod factors in the vicinity of the plant's root hairs that induce root hair deformation and the induction of cortical cell divisions within the root forming the nodule primordia. The bacteria become enclosed in an infection thread and ultimately infect cells in the core of the nodule by endocytosis (reviewed in [25,26]).

In the mature nodule, the rhizobia bacteroids are found in a specialized enlarged "infected cell" within the core of the root nodule. The bacteroids are enclosed in a membrane of plant-derived origin termed the symbiosome membrane [27], forming the fundamental nitrogen-fixing organelle, the symbiosome. Infected cells are generally nonvacuolated and the symbiosome constitutes the major organelle (Fig. 1). The symbiosome membrane is derived from the plasma membrane, but has characteristics of both plasma and vacuolar membranes [28]. The symbiosome membrane controls all metabolic traffic between the plant cytosol and the enclosed endosymbiont, as well as protects the bacteria from plant defense responses (reviewed in [23,24]). A summary of the transport activities on the symbiosome membrane is summarized in Table 1.

Nodule development is accompanied by the temporally and spatially-regulated expression of plant genes that encode proteins termed "nodulins" [29] which are involved in various aspects of nodule development and the establishment and maintenance of the symbiosis (reviewed in [25]). Among these nodulins are several symbiosome membrane-associated proteins [30–34], a number of which perform transport functions on this specialized symbiotic interface. Soybean nodulin 26 was originally discovered as a major protein targeted to the symbiosome membrane by the work of Fortin
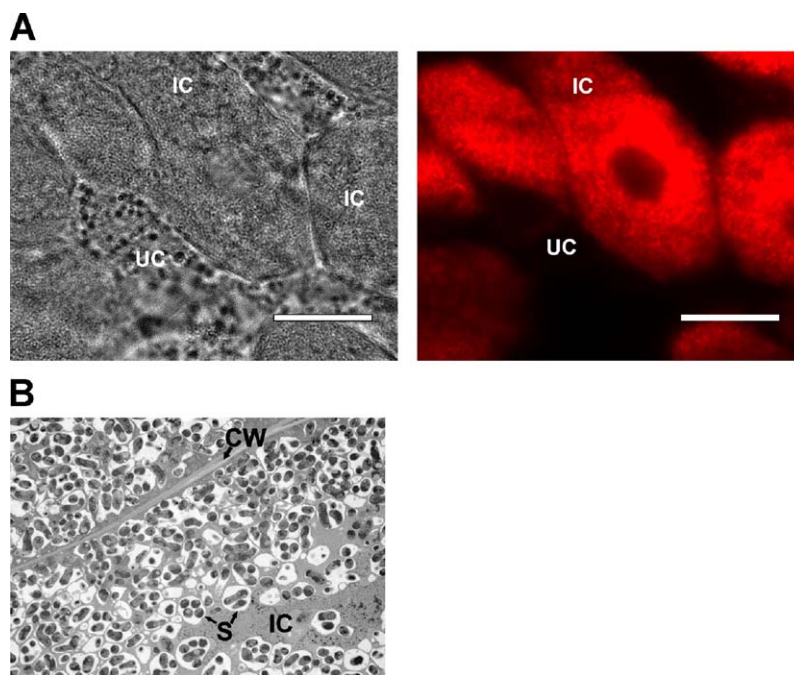

Fig. 1. Nodulin 26 is localized to the symbiosome membrane of infected cells. (A) The left panel shows a bright field image of a sectioned 28-day-old soybean nodule with infected cells (IC) and an uninfected cell (UC) indicated. The right panel shows the same nodule section probed with the nodulin 26 C loop antibody (from [83] with permission of the American Society of Plant Biologists). Size bars represent 20 μm. (B) An electron micrograph of a 28-day-old soybean nodule infected cell cytosol (IC) is shown with the cell wall (CW), and bacteroid-filled symbiosomes (S) indicated.

Table 1
Transporter activities on the symbiosome membrane

Transporter	Transport activities	Reference
GmN70	Chloride, nitrite, nitrate	[83]
Non-selective cation channel	Ammonium, potassium, calcium	[47]
H^+-ATPase	Protons (ATP dependent)	[84]
Nodulin 26	Water, glycerol, ammonia	[11,12]
GmZIP1	Zinc	[85]
Ca^{2+}-ATPase Calcium	(Magnesium, ATP dependent)	[86]
GmDMT1	Fe^{3+}, Mn^{2+}, Cu^{2+}, Zn^{2+}	[87]
Dicarboxylate transporter	Malate, succinate	[88]

et al. [35]. Subsequently, it was noted that the protein possessed homology to bovine lens MIP (AQP0) and it was among the first MIPs discovered in higher plants [36,37]. Further work showed that the protein is only expressed in the infected cell [38]. Within the infected cell, nodulin 26 is localized exclusively to the symbiosome membrane where it constitutes as much as 15% of the protein and is a target for phosphorylation by a symbiosome membrane-associated, calcium-dependent protein kinase [39–41]. The regulatory effects of phosphorylation will be considered in a separate section below.

Expression of nodulin 26 was found to coincide with a rapid burst of membrane biosynthesis that precedes endocytosis and development of the symbiosome membrane [35,41]. Given the numerous functional activities associated with the symbiosome, and the selective biosynthesis and targeting of nodulin 26 to the symbiosome membrane, a transport role supporting the symbiosis has been proposed. The transport activity of nodulin 26 has been the subject of extensive biochemical and biophysical characterization in *Xenopus laevis* oocytes as well as in purified symbiosome membrane vesicles and reconstituted proteoliposomes [11–13,41,42]. From these analyses it is clear that nodulin 26 possesses aquaporin activity with a low unitary conductance compared to robust aquaporins such as mammalian aquaporin 1 (Table 2). Nevertheless, the high concentration of nodulin 26 confers upon the symbiosome membrane a high osmotic water permeability (P_f=0.05 cm/sec) which shows the hallmarks of protein facilitated water permeability, including a low activation energy (3–4 kcal/mol) and sensitivity to the classical aquaporin inhibitor $HgCl_2$ [11,12] as well as to heavy metals such as $AgNO_3$ and $HAuCl_4$ [43]. Besides permeability to water, nodulin 26 was found to be multifunctional, showing permeability to test solutes such as formamide and glycerol [11,12], as well as ammonia [13], and was one of the first aquaglyceroporins documented in plants.

Nodulin 26 homologues have been isolated on the symbiosome membranes of other legumes including the genetic models *Medicago truncatula* [34] and *Lotus japonicus* [17], suggesting that the protein likely plays a conserved role during the symbiosis. However, in light of the multifunctional nature of nodulin 26 transport, the elucidation of this role has been difficult. Given the high water permeability of the symbiosome, and the fact that this is the major organelle in the specialized infected cell, a potential role in osmoregulation and sensing has

been proposed [11,12,41]. Thus, the high water permeability of symbiosome membrane conferred by nodulin 26 could serve a role in regulation of cytosolic volume homeostasis and osmotic regulation, similar to the proposed role of TIPs on the tonoplast of the central vacuole of other plant cells (reviewed in [44]). Additionally, given the sensitivity of the nodule to osmotic stress signals, and the observation that stress stimulates the phosphorylation of nodulin 26 [41], another potential function could be osmotic adaptation to external salinity and/or drought stress.

The biological significance of the glycerol transport behavior of nodulin 26 is less well understood since there is no apparent role for glycerol transport in metabolic support of the symbiosis or for a role in osmoregulation (similar to the glyceroporin FPS in yeast [45]). However, given the importance of symbiosome membrane in metabolic exchange during the symbiosis, the proposed function of nodulin 26 in facilitated transport of NH_3 in symbiosome membrane vesicles [13] is potentially significant. The transport mechanism for fixed nitrogen across the symbiosome membrane is still a subject of debate with transport mechanisms for uncharged ammonia [13,46] and for the charged NH_4^+ species (via an inwardly rectifying, voltage-sensitive symbiosome membrane cation channel [47–49]) being proposed. Both activities provide discreet pathways for reduced nitrogen efflux that may depend upon the pH of the symbiosome space (discussed in 13). Both mammalian AQP1 and AQP8 [14,50] as well as members of the Arabidopsis TIP family [14,15] transport ammonia, indicating that there is a precedent for transport of this uncharged metabolite by MIPs. The observation of a facilitated NH_3 transport activity on the symbiosome membrane suggests that this NIP transport property may have been recruited for ammonia transport in nitrogen fixing symbioses.

In support of a potential function of nodulin 26 in ammonia transport and assimilation, we have recently found that the cytosolic carboxyl-terminal domain (which contains the phosphorylation epitope [41]) of nodulin 26 provides a site for interaction with soybean glutamine synthetase (I.S. Wallace and D.M. Roberts, unpublished data). Glutamine synthetase catalyzes the ATP-dependent ligation of glutamate and ammonia, and represents the first committed step to nitrogen assimilation and utilization by the plant (reviewed in [51]). Considering the potential toxicity of excess ammonia in

Table 2
Transport properties of NIPs and AQP1

Protein	Unitary conductance (cm^3/s)	Transported solutes	E_a (kcal/mol)
Nodulin 26	0.38×10^{-14}	water, glycerol, formamide, ammonia	4.07
AtNIP6;1	n.d.	glycerol, formamide, urea	–
AQP1	11.4×10^{-14}	water, ammonia	2.20

A table summarizing the transport selectivity and properties of nodulin 26 and AtNIP6;1 compared to aquaporin 1. The unitary conductance for water transport of nodulin 26 [12] and AQP1 [89] is given along with a list of transported solutes for each protein. In addition, the activation energy for water transport is given.

the cytosolic compartment [52], the close association of glutamine synthetase with nodulin 26 at the cytosolic surface of the symbiosome may serve to facilitate the rapid assimilation of transported ammonia, preventing its accumulation.

3. Structural diversity and phylogeny of the NIP family

Although considerable initial focus has been placed on soybean nodulin 26, it has become evident that NIPs are widely distributed in both leguminous and nonleguminous plants, with multiple members found in every sequenced higher plant genome [1–4] (Fig. 2). This observation indicates that plant NIP function is not limited to the role that they play in nodule symbiosis. Indeed, members of this subgroup have been found in nonvascular plant species such as the moss *Physcomitrella patens* [53] suggesting that plants developed a need for NIPs early in land plant evolution, and it has been proposed that the origin of NIPs in plants occurred by a vertical gene transfer of a microbial glyceroporin [54].

The first NIPs characterized from nonleguminous plants were Arabidopsis NIP1;1 and NIP1;2 [16,55]. Similar nodulin 26-like gene products were subsequently identified from the suspensor ligament of loblolly pine embryos [18] as well as seed coats of pea [19]. Similar to nodulin 26, all of these genes have been shown to encode functional aquaglyceroporins. While these observations support the general model for structural and functional homology among the NIP subfamily, phylogenetic analysis suggests the presence of different NIP subgroups [1–4]. For example, analysis

of Arabidopsis NIPs shows the presence of seven phylogenic groups [1,2], whereas analysis of the Rice and Corn NIP families shows four [3] and three [4] NIP subgroups, respectively. Although structural information is not available for NIPs, the recent atomic resolution crystal structures of several other MIPs [56–58] and homology modeling techniques have allowed us to form hypotheses about how structural differences in the various NIP subgroups are related to their unique functional properties [8,59]. As discussed below, our analysis suggests that NIPs can be divided into two pore "families" with distinct functional properties.

MIPs have a conserved membrane topology fold consisting of six membrane spanning alpha helices that are interrupted by five loops (loops A–E), and cytosolic N- and C-terminal extensions (Fig. 3A). Loops B and E contain the highly conserved asparagine–proline–alanine (NPA) boxes, and form helices that fold back into the core of the protein to form one of the two major constrictions of the pore, the NPA region (Fig. 3B). About 8 Å above the NPA region is a second constriction region that is referred to as the aromatic/arginine (ar/R) region [60] because of the high prevalence of aromatic and basic residues. The ar/R is composed of four amino acid residues, one each from helix 2 (H2) and helix 5 (H5), as well as two residues from loop E (LE$_1$ and LE$_2$) (Fig. 3). Various biochemical, structural, and computational studies have highlighted the importance of this region in forming the selectivity filter of the MIP pore [8,42,56–61].

Homology modeling of the Arabidopsis NIP subfamily (nine full-length genes) suggests that the overall fold and
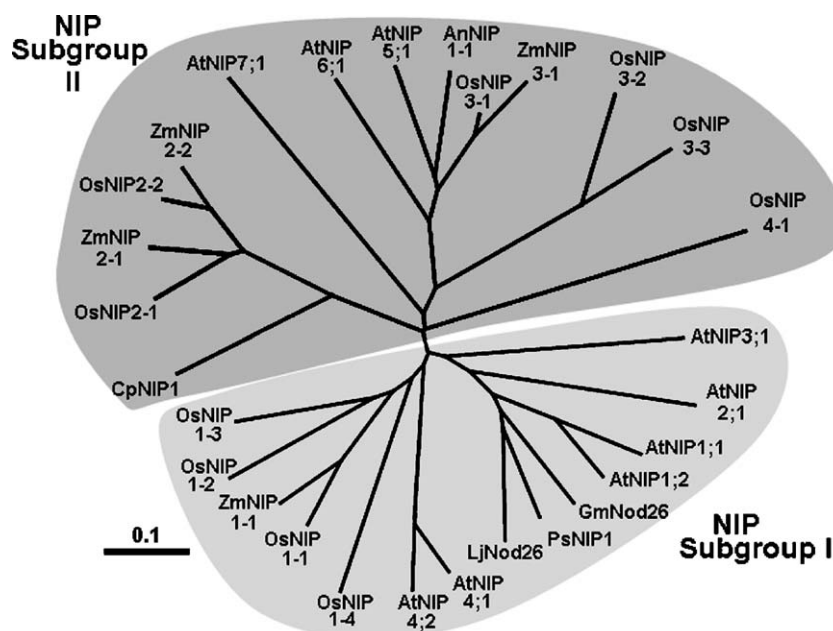

Fig. 2. NIP subfamily phylogeny: Phylogenetic analysis of the deduced amino acid sequences of selected NIP family members from various plant species. The division between NIP subgroup I and II is indicated. Accession numbers used in the initial sequence alignment are as follows: GmNod26 (soybean nodulin 26) (JQ2285), LjNod26 (AF275316), AnNIP1-1 (AF544251), AnNIP1-2 (AF544252), OsNIP1;1 (BAD27715), OsNIP1;2 (BAD73177), OsNIP1;3 (AAV44140), OsNIP1;4 (BAD53665), OsNIP2;1 (BAD16128), OsNIP2;2 (BAD37471), OsNIP3;1 (AAG13499), OsNIP3;2 (BAC99758), OsNIP3;3 (BAC65382), OsNIP4;1 (BAB61180), ZmNIP1-1 (AF326483), ZmNIP2-1 (AF326484), ZmNIP2-2 (AF326485), ZmNIP3-1 (AF326486), AtNIP1;1 (Q8VZW1), AtNIP1;2 (Q8LFP7), AtNIP2;1 (CAC81707), AtNIP3;1 (Q9C6T0), AtNIP4;1 (Q9FIZ9), AtNIP4;2 (Q8W036), AtNIP5;1 (Q9SV84), AtNIP6;1 (Q9SAI4), AtNIP7;1 (Q8LAI1), PsNIP1 (CAB45652). Multiple sequence alignments were done by using Clustal W version 1.83 (part of the MegAlign program of the DNAStar Lasergene suite) using the slow, accurate setting and the Gonnet 250 matrix (open gap penalty set to 10.0). The unrooted phylogenetic tree shown was generated from these alignments by using SeqTools version 8.2.102. The bottom bar length represents 0.1 substitutions per amino acid.
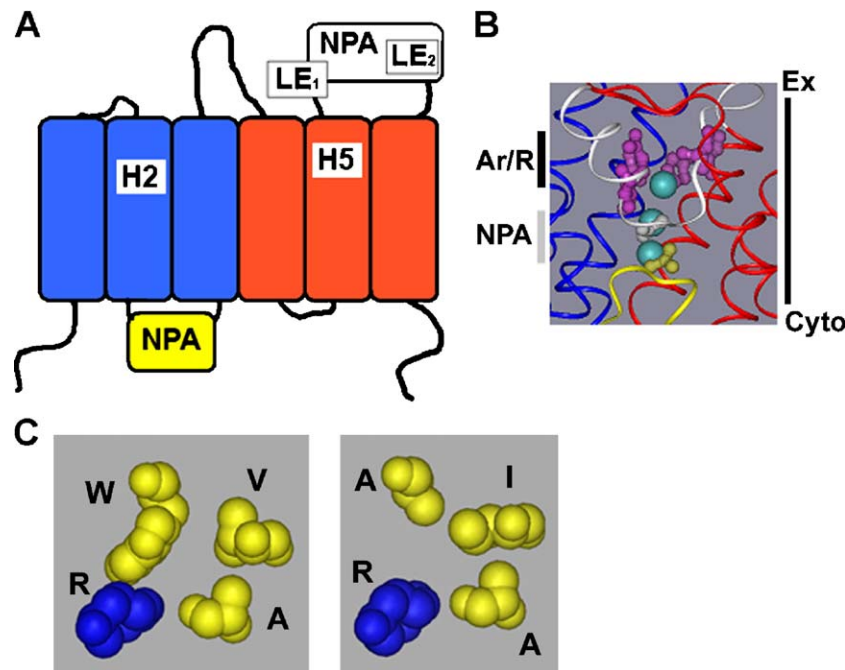
Fig. 3. Major Intrinsic Protein structure and the aromatic/arginine selectivity filter. (A) The MIP conserved topology is shown with the first three transmembrane helices in blue and the last three in red to illustrate the pseudo two-fold symmetry present in the molecule. The highly conserved NPA motifs are shown as boxes with the N-terminal NPA shown in yellow, and the C-terminal NPA shown in white. The relative positions of the ar/R selectivity filter residues H2 (helix 2), H5 (helix 5), LE$_1$, and LE$_2$ (loop E 1 and 2 positions) are shown. (B) The experimental X-ray structure of bovine AQP1 (PBD ID: 1J4N; [57]) is shown with the ar/R selectivity region (magenta) and the two NPA asparagines (white and yellow) indicated. Transmembrane helices are shown in blue and red corresponding to Panel A. Bound waters are shown as blue spheres. The bar to the side of the structure indicates the relative position of the extracellular space and the cytosol. (C) The two NIP ar/R signatures of subgroup I (nodulin 26) and subgroup 2 (as represented by AtNIP6;1) are shown in the following order: clockwise, position H2, H5, LE$_1$, and LE$_2$. The residues are color coded as follows: hydrophobic residues, yellow and basic residues, blue. Reproduced from [8] with the permission of the American Society of Plant Physiologists.

topology of these proteins are likely conserved [8]. However, an examination of the modeled ar/R regions of Arabidopsis NIPs shows that they can be divided into two ar/R subgroups: NIP I and II proteins, which have unique properties that are conserved within each subgroup [8]. Arabidopsis NIP subgroup I contains 6 members (AtNIP1;1, AtNIP1;2, AtNIP2;1, AtNIP3;1, AtNIP4;1, and AtNIP4;2) that resemble nodulin 26 at the ar/R region. These NIPs have a conserved tetrad of residues with a Trp at H2, Val/Ile at H5, Ala at LE$_1$, and Arg at LE$_2$ [8]. The ar/R region of NIP subgroup I is a hybrid of aquaporin and glyceroporin-like residues and is proposed to determine transport rate as well as selectivity [42,59]. The Arg at LE$_2$ are proposed to form the hydrogen bond contacts that are necessary to allow passage of glycerol, while the Trp, Val and Ala residues serve to increase the hydrophobicity of the ar/R region to form van der Waals contacts with glycerol hydrocarbon backbone while simultaneously increasing the diameter of the putative pore to allow passage of the larger solute [42]. Consistent with this hypothesis, site directed mutagenesis of Trp at the H2 of LjNod26 with a hydrophilic His residue (characteristic of TIPs) abolished the transport of glycerol but left water transport properties unaffected [42]. Transition state analysis of water transport through nodulin 26 also supports this proposed model of the nodulin 26 ar/R region because nodulin 26 has one less hydrogen bond acceptor available at the ar/R

region than AQP1, and the enthalpy of the transition state for water transport between these protein differs by the energy of a hydrogen bond [59].

NIP subgroup II in Arabidopsis contains three members (AtNIP5;1, AtNIP6;1, and AtNIP7;1) which all contain a nonconservative ar/R amino acid substitution of Ala at H2 for Trp in subgroup I. Homology modeling of the NIP II pore using AtNIP6;1 as a test case shows that this substitution results in a marked increase in the predicted pore diameter [8,59]. Interestingly, recent functional characterization of NIP subgroup II show that this H2 substitution is responsible for a distinct transport selectivity compared to NIP subgroup I transporters. AtNIP6;1, a representative type II NIP, shows high permeability to glycerol, similar to NIP subgroup I, but is completely impermeable to water [59]. Also, consistent with their larger pore aperture, AtNIP6;1 is permeable to larger solutes such as urea, which is excluded by nodulin 26 [11,59]. The reason for the low water permeability of NIP subgroup II members despite their large pore size is intriguing. This property could conceivably arise from several different sources: gating phenomena in the actual pore, such as the type that occurs in GlpF [62], gating by cytosolic termini as demonstrated in the yeast FPS glyceroporin [63], or lack of the ability to organize transported water in the pore [57].

Outside of Arabidopsis, members of NIP subgroup II have been identified in several plant species including corn [4], rice

[3], *Atriplex nummularia* [20], and cucumber [21] indicating that NIP II proteins are common to most plants. Analysis of the NIP subfamily phylogeny shows that the two pore families segregate into distinct clades (Fig. 2). Similar to the NIP subgroup I, the biological functions of NIP subgroup II remain unzclear. However, the divergence in the selectivity of NIP II channels and the low permeability to water suggest that these proteins likely have physiological roles distinct from the water transporting NIP I proteins. The high urea permeability of NIP subgroup II members is intriguing since this is a physiological solute in plants [64] that is produced during the degradation of amino acids during seed germination and embryo development [65]. Whether NIP II transporters play a role in the transport of this solute, or other potential uncharged metabolites in plants, merits further consideration.

4. Regulation of NIPs: phosphorylation

It has become clear that both animal and plant MIPs are often targets for protein phosphorylation within their cytosolic termini and loop regions (reviewed in [66]) with such modifications leading to modulation of transport function [67,68] and/or regulation of trafficking and localization to various cellular locations [69]. NIPs are no exception. Indeed, even before its transport function was understood, nodulin 26 was initially detected in symbiosome membranes as a major target of calcium-dependent phosphorylation [39]. Phosphorylation of nodulin 26 occurs within the cytosolic C-terminal domain at Ser 262, and is catalyzed by a symbiosome membrane associated Calcium Dependent Protein Kinase (CDPK) [39]. By using antibodies that specifically recognize the phosphorylated epitope of nodulin 26, phosphorylation was shown to be regulated in vivo according to the developmental state of the nodule as well as in response to osmotic stress signals [41]. Nodulin 26 protein levels accumulate during the endocytosis/infection state of nodule development, accompanying the burst of membrane biosynthetic activity that precedes symbiosome formation. However, phosphorylation levels lag behind protein synthesis and become prevalent upon nodule maturation and remain at a steady state level during the period of active nitrogen fixation (Fig. 4).

Abiotic stresses such as salinity and drought were shown to increase phosphorylation further during this stage. In light of the finding that phosphorylation of ser 262 stimulates the water transport rate of nodulin 26 (Fig. 4 [41]), two modes of regulation are possible. During normal nitrogen fixation,
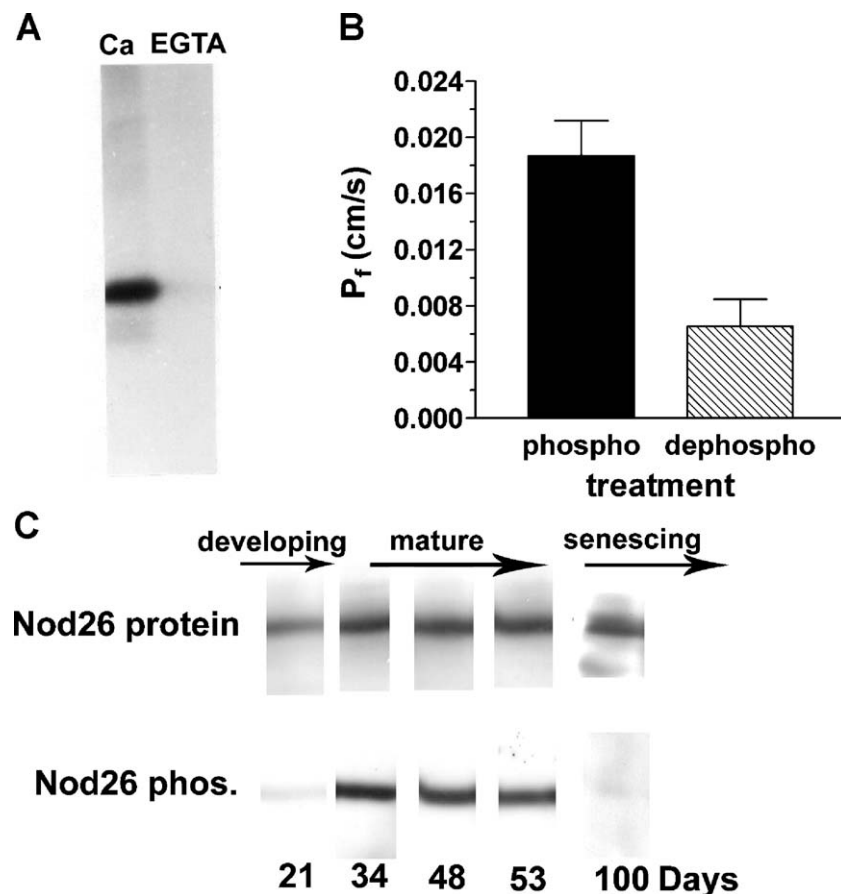

Fig. 4. Nodulin 26 is regulated by calcium dependent phosphorylation: (A) An autoradiogram of purified symbiosome membranes treated with either CaCl$_2$ or EGTA in the presence of γ [^{32}P] ATP. The arrow to the right indicates the position of nodulin 26. (B) The effect of phosphorylation on nodulin 26 water permeability was measured by stopped flow fluorimetry of CDPK phosphorylated or phosphatase-treated nodulin 26. (C) Analysis of the appearance of nodulin 26 protein and nodulin 26 phosphorylation during nodule development using specific antibody probes in a Western blot analysis. Samples were taken during a nodule developmental time course from 21 to 100 days post-germination. From [41] with permission of the American Society of Plant Physiologists.

phosphorylation could represent a means of regulating the water and solute permeability of the symbiosome membrane during times of high metabolic activity to maintain an osmotic balance. Secondly, in conditions of severe osmotic stress, nodulin 26 may play an additional role in adaptation of the infected cell to these stresses [41].

Many NIP subgroup 1 proteins from Arabidopsis, corn, and rice contain consensus CDPK phosphorylation sites (hydrophobic-X-basic-X-X-Ser/Thr) within their C-terminal extensions similar to nodulin 26 (Table 3). CDPKs are involved in a wide range of physiological processes in the plant including Ca^{2+} signaling during bacterial initiation of the nitrogen fixation symbiosis, biotic and abiotic stress adaptation, pollen development, nitrogen metabolism, and hormone signaling (reviewed in [70–72]). Thus, there appears to be significant overlap between processes that may involve NIPs and many of the processes that are regulated by CDPKs. The conservation of the CDPK site in the C-terminus suggests that NIP subgroup I regulation by CDPKs may be typical of the group. The question of the regulatory effects of NIP phosphorylation still remains somewhat of an open question. It is of interest to note from the recent solution of the structures of open and closed SoPIP2;1 [73] that gating of the protein appears to be modulated at least in part by phosphorylation of Ser 274 at an analogous location within the C-terminal domain. Given the activation of nodulin 26 by phosphorylation, a similar gating mechanism may be in place for NIP proteins. In addition to modulation of changes in activity, other potential roles of phosphorylation need to be considered. For example, phosphorylation of mammalian AQP2 within an analogous location in the carboxyl terminal region does not affect transport activity, but is critical for trafficking changes (reviewed in [69]). Further, as discussed above, the phosphorylation site of NIPs could also represent a location for the binding of
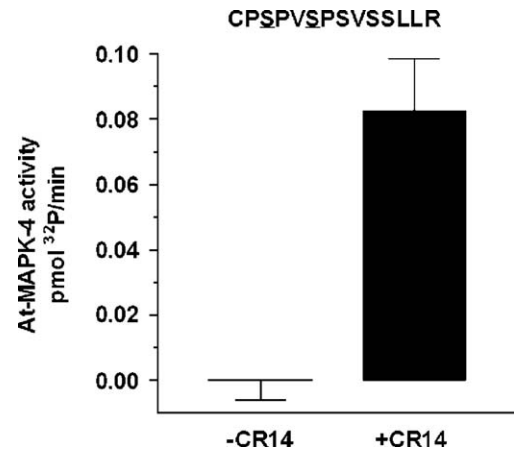

Fig. 5. AtNIP7;1, a NIP subgroup II member, is phosphorylated by MAP kinase. The ability of a peptide (CR-14) with the C-terminal sequence of AtNIP7;1 (sequence shown) to serve as an in vitro substrate for purified activated AtMPK4 [91] was tested. The histogram shows the incorporation of ^{32}P-phosphate into samples of 750 μM CR-14 by AtMEK1 in the presence of γ [^{32}P] ATP with error bars showing the S.E.M.

auxiliary proteins and their localization to a particular membrane location.

Less is known about regulation of NIP subgroup II members by phosphorylation. Typically, these proteins are divergent from NIP I proteins at their termini and lack a conserved CDPK site in the C-terminal domain. However, several have a more extended N-terminal region, and many have consensus Mitogen Activated Protein Kinase (MAPK) sites (Pro-X-Ser/Thr-Pro) within the cytosolic C- or N-terminal extensions (Table 3). Experiments with the C-terminal extension of AtNIP7;1, an Arabidopsis NIP subgroup II member, show that this site is phosphorylated by activated AtMPK4 in vitro (Fig. 5). MAPKs are involved in plant stress responses and hormonal signaling [74,75], and whether MAPK signaling pathways might play in the regulation of NIP subgroup II needs to be investigated further.

5. Regulation of NIPs: gene expression

While it is clear that nodulin 26 is a symbiosis-specific protein, the larger question of NIP expression and function in general plant physiology still remains open. Analyses of NIP expression in model organisms such as Arabidopsis [22], rice [3] and maize [4] suggest that NIP genes in general are expressed at a much lower level compared to most PIPs, TIPs and SIPs. Nevertheless, microarray data shows interesting trends and tissue specific expression (Table 4). For example, AtNIP7;1, 4;1 and 1;2 appear to be largely expressed in flower tissues, whereas AtNIP6;1 is predominantly expressed in stems, and AtNIP1;1 appears to be root specific (Table 4), suggesting these proteins may play specific roles in these tissues. Further analysis shows that expression is often temporally and spatially regulated. For example, in the case of AtNIP7;1, which encodes a NIP subgroup II protein, Q-PCR results show 50 fold higher levels of transcript in floral tissue compared to other Arabidopsis tissues, and microarray analysis shows that

Table 3
Conserved phosphorylation sites in the N- and C-termini of NIPs

Gene	Peptide sequence	Predicted kinase	NIP subgroup member
AtNIP1;1	ITKSGS**S**FLK	CDPK (C)	Subgroup I
AtNIP1;2	ITKSGS**S**FLK	CDPK (C)	Subgroup I
AtNIP2;1	FSKTGS**S**HK	CDPK (C)	Subgroup I
AtNIP4;1	LTKSAS**S**FLR	CDPK (C)	Subgroup I
AtNIP4;2	LTKSAS**S**FLR	CDPK (C)	Subgroup I
AtNIP5;1	PPTPG**T**PGTPGGP	MAPK (N)	Subgroup II
AtNIP6;1	PS**T**PSTPATTPGTP	MAPK (N)	Subgroup II
AtNIP7;1	RPCPSPV**S**PS	MAPK (C)	Subgroup II
ZmNIP1;1	ITKST**S**FLK	CDPK (C)	Subgroup I
ZmNIP3;1	PPNGSAPA**T**PGTPA	MAPK (N)	Subgroup II
OsNIP1;1	ITKLSGS**S**FLK	CDPK (C)	Subgroup I

A table showing a list of putative phosphorylation sites of NIPs from deduced amino acid sequences in the rice, corn, and *Arabidopsis thaliana* genomes. The gene names are given according to previously published phylogenetic data [1–4]. The putative phosphorylation epitope is shown with the predicted phosphorylated residue in bold type. Kinases that are predicted to phosphorylate these sites are also given with the consensus phosphorylation sequences as follows: CDPK; hydrophobic-X-basic-X-X-Ser/Thr, and MAPK; P-X-S/T-P. The location of the phosphorylation site (N or C terminal regions) is given in parenthesis. The NIP subgroup of each gene is also reported according to the nomenclature of [8].

Table 4
Organ expression of Arabidopsis NIPs

Tissue	NIP1;1	NIP1;2	NIP2;1	NIP3;1	NIP4;1	NIP5;1	NIP6;1	NIP7;1
Flower	131 (3.29)	2410 (9.23)	119 (1.60)	155 (5.01)	665 (20.6)	753 (12.8)	372 (19.0)	2180 (111)
Silique	56.0 (6.51)	656 (54.2)	113 (18.1)	64.1 (8.19)	84.6 (8.75)	1050 (62.3)	615 (78.3)	259 (20.1)
Stem	49.2 (21.2)	314 (31.5)	95.9 (6.0)	49.0 (4.82)	72.7 (7.70)	670 (58.3)	3890 (103)	222 (21.5)
Leaf	140 (8.14)	560 (48.0)	114 (38.2)	56.0 (8.96)	147 (2.99)	117 (51.0)	241 (15.4)	361 (55.3)
Root	3260 (380)	859 (24.9)	118 (26.2)	94.4 (11.3)	138 (14.9)	2290 (157)	197 (35.5)	184 (25.8)

Data were obtained from the Digital Northern database from Genevestigator [90] and represent microarray results from AtGenExpress (exp 92 Plant Organs). The signal of 3–6 replicates is shown with the S.E.M. in parenthesis.

AtNIP7;1 upregulation appears to be specific for floral stage 9, with a decrease in expression observed through the floral stage 10 to 12 (Fig. 6). During stage 9, all organs, especially petals, stamens, and gynoecium of the flower, undergo a rapid lengthening and extended expansion growth, and this stage is the most important and longest among the 12 early stages of floral development [76]. Similarly, analysis of AtNIP6;1 in stem tissues suggest that this protein is selectively expressed at stem nodes (data not shown). Collectively, these observations suggest that the physiological need for several of these NIPs
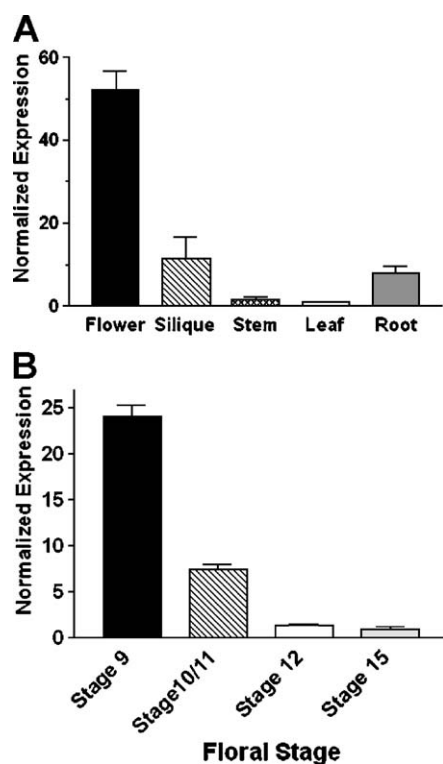

Fig. 6. Expression analysis of AtNIP7;1: (A) RNA was extracted from the flower, siliques, stem, leaf, and root tissues of 6-week-old Arabidopsis plants (ecotype Columbia) grown under a long day (LD) light cycle (16 hr light/8 hr dark). 4 μg of RNA was transcribed to cDNA, and AtNIP7;1 was amplified from 10 ng of the resulting cDNA samples by Q-PCR using gene specific primers for AtNIP7;1. The data were then normalized to the leaf signal. Error bars show S.E.M. ($n = 5$ for flower, stem, leaf, and root; $n = 2$ for siliques). (B) Microarray (Digital Northern) data of AtNIP7;1 expression using an affy 22 K chip and cDNA from different floral stages was obtained from Genevestigator (http://www.genevestigator.ethz.ch/ [90]). The data were normalized to floral stage 15 signal. Error bars show the S.E.M. ($n = 3$).

is transient and possibly organ or cell specific, similar to the original findings with the subfamily archetype in soybean, nodule-specific nodulin 26. Observations from other plant models support this trend. For example, in the embryos of the loblolly pine, a NIP subgroup I member (PtNIP1;1) is specifically expressed in the suspensor ligament of the embryo, and the expression level of this gene rapidly declines upon embryo maturation [18,77]. PsNIP1;1, a NIP subgroup I-like aquaglyceroporin, is expressed in the developing seed coats of pea seeds, but not in roots, shoots, or cotyledons, in a developmentally specific manner [19].

Plant major intrinsic proteins are typically highly regulated at the transcriptional level, responding to a diverse array of developmental and stress related signals [78] and the NIPs are no exception [22]. For example, AtNIP1;1 was shown to be down severely down-regulated under a variety of osmotic stresses such as drought and salinity, as well as during treatment with the stress hormone abscisic acid [55]. Along with the observation that osmotic stresses affect the phosphorylation and transport activities of NIPs [41], these observations suggest that modulation of NIP activity and/or expression is part of the coordinated response of plants to osmotic challenge.

6. Conclusions and future prospects

Nodulin 26 is one of the first MIPs identified in plants, and it is clear that the NIP family represents a unique clade of plant-specific major intrinsic proteins. While the family has been subject to considerable biochemical and biophysical analysis, the biological role of NIPs is still a subject of debate. Further understanding of these roles will require an intimate knowledge of the cellular and subcellular localization of NIPs, and clarification of their transport properties as well as the factors that mediate both genetic and posttranslational regulation. The cellular and subcellular localization of NIPs in soybean nodules is well characterized. However, the localization of NIPs in non-legumes, which have no real parallel to the symbiosome membrane, is less clear. Heterologous expression of nodulin 26 in transgenic tobacco results in targeting to the tonoplast membrane [79], but these conditions do not represent a native system so it is difficult to determine whether this is the normal membrane within which NIPs reside.

An emerging area with respect to NIPs, and MIPs in general, is the role of protein–protein interactions with various metabolic and regulatory proteins at the membrane–cytosol interface. While the effects of phosphorylation, Ca^{2+}, pH, and

several other regulatory mechanisms have been well studied (reviewed in [66]), investigation of the functional significance of protein–protein interactions in plant MIP function is still in its infancy. There are some obvious examples that could serve as models for other systems. For example, human AQP2 has been shown to interact with numerous proteins involved in membrane trafficking [80], while the *E. coli* glycerol facilitator GlpF has been shown to interact and increase the activity of glycerol kinase in vitro [81] serving as a metabolic funnel. Thus protein–protein interactions obviously play an important and understudied role in MIP biology.

Even with this library of data available, elucidation of the in vivo function of NIPs will require the use of genetic knockout or knockdown technologies to assess the global effect of these proteins on plant development and environmental responses. The large collection of Arabidopsis T-DNA knockouts and improved silencing technologies for model legumes [82] may provide the tools to begin to more fully understand the roles that NIPs play in legume symbioses as well as a general role in nonleguminous plants.

Note added in proof

Since the submission of this review two exciting studies have appeared or are in press that provide new insight into the transport functions of NIP II subgroup proteins in the transport of nutritionally important metalloid compounds. In the first study, Ma et al. [92] have shown that the OsNIP2;1 protein is a silicon transporter encoded by the Low silicon rice 1 gene. This NIP is essential for silicon uptake by rice roots, and mutants with defective LsrI show increased susceptibility to pests and diseases. In a second study, Takano et al. [93] show that the AtNIP5;1 protein is a boric acid transporter which is induced in Arabidopsis plants under conditions of limiting boron. The authors have shown that T-DNA knockouts of AtNIP5;1 show normal growth when boron is sufficient, but show dramatic developmental sensitivity to boron limiting conditions, suggesting this NIP II protein is essential for boron uptake under these conditions. These findings further underscore the theme that MIP proteins in plants have diverged to provide additional functions besides water transport.

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