To the Graduate Council:

I am submitting herewith a dissertation written by Charles David Weaver entitled "Structural and Functional Characterization of the Nodulin 26 Ion Channel and the Calmodulin-like Domain Protein Kinase from Soybean Root Nodules." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biochemistry.

Daniel M. Roberts, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor
and Dean of The Graduate School

STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF THE NODULIN 26 ION CHANNEL AND THE CALMODULIN-LIKE DOMAIN PROTEIN KINASE FROM SOYBEAN ROOT NODULES

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ABSTRACT

Nodulin 26 is a nodule-specific integral membrane protein proposed to be found in the symbiosome membrane of nitrogen-fixing soybean root nodules (Fortin *et al.*, 1987; Sandal and Marker, 1988). In this study, nodulin 26 protein has been detected in nitrogen-fixing soybean root nodules by using specific immunological probes. These probes have been utilized to verify that the nodulin 26 protein is specifically expressed in nodule tissue. Furthermore, immunoelectron microscopy, as well as Western blot analysis of isolated membrane fractions, have been used to demonstrate that nodulin 26 is found only in the SM of the infected cells of nitrogen-fixing nodules. In soybean nodules that are infected by mutant rhizobium that are defective in release into symbiosomes, nodulin 26 was expressed at levels comparable to wild-type nodules. Taken together, these data demonstrate that nodulin 26 is a nodule-specific protein that is localized to the symbiosome membrane and that the control of nodulin 26 expression is not contingent upon the release of bacteria into symbiosomes nor upon the establishment of a nitrogen-fixing symbiosis.

Soybean root nodules were found to contain a calcium-dependent protein kinase activity that phosphorylated a synthetic peptide (CK-15) containing a sequence found within the 14 carboxyl terminal residues of nodulin 26. This protein kinase activity was purified from the soluble fraction of

nodules. A similar activity was partially purified from symbiosome membranes. The soluble protein kinase was activated by μM Ca^{2+}. Neither maximal activity nor the ability to respond to Ca^{2+} were dependent on calmodulin or lipid effectors. These observations suggest that this enzyme is a member of the calmodulin-like domain protein kinase family. This is supported by Western blot analyses that show that the nodule protein kinase crossreacts with monoclonal antibodies against the calmodulin-like domain protein kinase. The symbiosome membrane-associated kinase was resistant to removal by chaotropic agents, such as KI, that are known to remove extrinsic membrane proteins. However, the activity could be solubilized by the zwitterionic detergent CHAPS and displayed 400-fold Ca^{2+}-stimulation after chromatography by Mono Q ion-exchange. Unlike the soluble protein, the symbiosome membrane-associated protein kinase did not react with monoclonal antibodies against the calmodulin-like domain protein kinase. Thus, the symbiosome membrane-associated protein kinase appears to be distinct from the soluble protein kinase and is integrally associated with the symbiosome membrane.

Ca^{2+}-dependent nodulin 26 phosphorylation was detected in symbiosome membranes *in vitro* and in intact nodules metabolically-labeled with ^{32}P-phosphate. The phosphorylation site of nodulin 26 was determined to be a single serine residue, ser^{262}, found in the carboxyl terminus of the

molecule. This site was also found within the CK-15 sequence. These data demonstrate that soybean root nodules contain protein kinase activities that are activated by physiologically relevant Ca^{2+} concentrations. Furthermore, these data demonstrate that nodulin 26 is phosphorylated on a single serine residue by a calcium-dependent symbiosome membrane-associated protein kinase. Therefore, nodulin 26 may be a target of Ca^{2+}-dependent regulation in soybean root nodules.

Observations that nodulin 26 phosphorylation correlates with malate transport rates in intact symbiosomes led to a direct investigation of the functional activity of nodulin 26. To accomplish this, nodulin 26 was purified to apparent homogeneity from soybean root nodules, reconstituted into liposomes, and assayed for ion channel activity in planar lipid bilayers. These studies demonstrated that nodulin 26 formed a high-conductance ion channel with a maximum unitary conductance of 3.1 nS. The channel exhibited only slight anion-selectivity based on reversal potential measurement. The nodulin 26 ion channel was voltage-sensitive with a tendency to occupy lower conductance states, or to close completely, in the presence of high voltage potentials (>60 mV). Preliminary experiments suggested that phosphorylation may confer voltage sensitivity on the nodulin 26 ion channel. Taken together, these data demonstrate that nodulin 26 can form ion channels in planar lipid bilayers and that

phosphorylation may be involved in modulating its activity. These observations support a role for nodulin 26 in symbiosome membrane transport and a role for Ca^{2+}-dependent phosphorylation in controlling this transport.

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LIST OF ABBREVIATIONS

bib, big brain; **β-ME**, β-mercaptoethanol; **BSA**, bovine serum albumin; **CCCP**, carbonyl-cyanide *m*-chlorophenyl hydrazone; **CDPK**, calmodulin-like domain protein kinase; **CHAPS**, 3-[(cholamidopropyl)dimethylammonio]-1-propane sulfonate; **CHIP**, channel-like intrinsic protein; **CK-15**, synthetic peptide based on the last 14 carboxyl-terminal amino acid residues of nodulin 26; **DEAE**, diethylaminoethyl; **DTT**, dithiothreitol; **EDTA**, ethylenediaminetetraacetic acid; **EGTA**, ethylene glycol bis(β-amino ethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid; **ELISA**, enzyme-linked immunosorbent assay; **ENOD**, early nodulin; ***FPS1***, fermentable sugar defect suppressor; ***GlpF***, glycerol facilitator; **HEPES**, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); **Lys-C**, endoproteinase Lys-C; **MBS**, *m*-maleimidobenzoic acid-*N*-hydroxysuccinamide ester; **MES**, 2-(*N*-morpholino)ethanesulfonic acid; **MIP**, major intrinsic protein; **MOPS**, 3-(*N*-morpholino)propanesulfonic acid; **NOD**, late nodulin; **OG**, 1-O-*n*-octyl-β-D-glucopyranoside; **ORF1**, open reading frame 1; **PAGE**, polyacrylamide gel electrophoresis; **PBS**, phosphate buffered saline (8 mM sodium phosphate, 1 mM potassium phosphate, pH 7.2, 0.14 M NaCl, 3 mM KCl); **PEG 8000**, polyethylene glycol (average MW = 8000); **PMSF**, phenylmethyl sulfonylflouride; **PVPP**, polyvinylpolypyrrolidone; **PVP-40**, polyvinylpyrrolidone (average MW = 40,000); **RIA**, radio-immunoassay; **SDS**, sodium dodecyl sulfate; **SM**, symbiosome membrane; **TES**, (*N*-tris[hydroxymethyl]-methyl-2-

aminoethanesulfonic acid); **TFA**, triflouroacetic acid; **TIP**, tonoplast intrinsic protein; **TLC**, thin-layer chromatography; **TLE**, thin-layer electrophoresis; **TPCK**, L-1-*p*-tosylamino-2-phenylethylchloro-methyl ketone; **Tris**, tris(hydroxymethyl)aminoethane; **TUR**, turgor inducible protein; **VU-1 calmodulin**, calmodulin derived from a synthetic gene (Roberts *et al.*, 1985).

CHAPTER I

INTRODUCTION

Calcium homeostasis and calcium-mediated signaling

Cells have evolved complex and energy expensive mechanisms to maintain low levels of cytosolic Ca^{2+} and thus protect themselves from the toxic effect of high intracellular calcium. Through the action of Ca^{2+}-pumping ATPases and the use of electrochemical gradients to drive Ca^{2+} transport, cells are able to maintain cytosolic Ca^{2+} levels at below μM concentrations (reviewed in Pietrobon *et al.*, 1990 and Bush, 1993). As a result of the ability of cells to maintain these low cytosolic levels of Ca^{2+}, they have evolved selective mechanisms that allow the levels of cytosolic Ca^{2+} to be transiently raised to from 10^{-7} to 10^{-6} M (Tsien, 1988). Cells have also evolved proteins that can sense and respond to these increases in cytosolic Ca^{2+} concentration (reviewed in Kretsinger, 1980; Strynadka & James, 1989; Moncrief *et al.*, 1990). Through these evolutionary mechanisms, cells have been able to turn the necessity to remove a toxic ion from their cytoplasm into an important signal transduction mechanism.

A major focus of plant signal transduction has been the investigation of what physiological, developmental, and environmental stimuli lead to Ca^{2+} fluxes. Using techniques similar to those that have been successfully used in animal systems, intracellular Ca^{2+} levels have been measured in

plants. Florescent Ca^{2+}-reporter dyes and proteins, such as aequorin, have allowed the measurement of the levels of Ca^{2+} in the cytosol of unstimulated and stimulated plant cells (reviewed in Read *et al.*, 1992). In unstimulated cells, cytosolic Ca^{2+} concentrations between 30 and 200 nM have been observed (reviewed in Read *et al.*, 1992). By using transgenic plants transformed with aequorin, effectors such as light, salt, fungal elicitors of plant defense responses, hormones, and mechanical stimuli have been observed to cause changes in cytosolic Ca^{2+} concentrations (Knight *et al.*, 1991; Knight *et al.*, 1992; Bush, 1993). These data demonstrate that plants are capable of maintaining low concentrations of cytosolic Ca^{2+} and that these Ca^{2+} concentrations can change in response to various physiological stimuli.

Clearly, cells appear to be able to sense cytosolic Ca^{2+} concentrations since cells injected with exogenous Ca^{2+} rapidly re-establish resting Ca^{2+} levels (Felle, 1988a; 1988b; Gilroy *et al.*, 1990). In animal systems, the proteins involved in the maintenance of Ca^{2+} homeostasis are well described (Pietrobon *et al.*, 1990), and more recently, some of the proteins believed to mediate the control of Ca^{2+} homeostasis in plants have been identified (reviewed in Bush, 1993). A diverse group of membrane-bound pump proteins drive the energy-dependent translocation of Ca^{2+} from the plant cell (reviewed in Bush, 1993). In addition to Ca^{2+} efflux mechanisms, Ca^{2+} influx channels have been identified by

electrophysiological measurements. By using patch clamp techniques, three types of Ca^{2+} channels have been observed: 1. voltage-sensitive Ca^{2+} channels have been identified associated with the plasma membrane and the tonoplast (Schroeder & Hagiwara, 1990; Fairely *et al.*, 1992; Johannes *et al.*, 1992; Pantoja *et al.*, 1993); 2. IP_3 activated channels have been described associated with the tonoplast (Alexandre *et al.*, 1993); and 3. stretch-sensitive channels have been observed in the plasma membrane of guard cells (Cosgrove & Hedrich, 1991).

Based on these data, it appears that plants, similar to animals, have energy dependent mechanisms for maintaining submicromolar levels of cytosolic Ca^{2+}. Furthermore, several Ca^{2+} channels have been identified that open in response to a variety of physiological stimuli. Opening of these channels leads to transient increases in cytosolic Ca^{2+} levels. When one considers all of the data supporting the importance of Ca^{2+} signal transduction in animal systems, it is not surprising that Ca^{2+} plays multiple, integral roles in plant growth and development. More uncertain, however, are the molecular details of Ca^{2+} signal transduction mechanisms in plants. In the following section proteins involved in sensing and responding to Ca^{2+} signals will be discussed.

Calcium-modulated proteins - targets of calcium signaling

The effects of elevations in cytosolic Ca^{2+} concentrations that allow Ca^{2+} to act as a second messenger

are mediated though proteins that can sense and respond to transient changes in Ca^{2+} concentrations (Kretsinger, 1980; Kwiatkowski *et al.*, 1986; Cohen & Klee, 1988; Nishizuka, 1988; Pietrobon *et al.*, 1990; Moncrief *et al.*, 1990). Perhaps the largest class of Ca^{2+}-sensor proteins to be described thus far is the EF-hand family of Ca^{2+}-binding proteins (Kretsinger, 1976; Kretsinger, 1980; Strynadka & James, 1989; Moncrief *et al.*, 1990). The members of this family can bind Ca^{2+} reversibly, with dissociation constants in the μM range under conditions of physiological ionic strength and pH, and thus are proposed to be targets of intracellular Ca^{2+} fluxes.

The Ca^{2+} binding motif common to these proteins is structurally conserved. Based on the first crystal structure of a member of this family, parvalbumin, it consists of two amphipathic α-helices which are found on each side of a 12 amino acid loop (Kretsinger & Nockolds, 1973). This arrangement constitutes a helix-loop-helix structure which has been termed an EF-hand which has been discussed in several reviews (Kretsinger, 1976; Kretsinger, 1980; Strynadka & James, 1989; Persechini *et al.*, 1989). This structure has been qualitatively described in terms of the E helix, an extended index finger; the Ca^{2+}-binding loop, a curled second finger; and the F helix, the thumb extended at a right angle with respect to the extended index finger (Kretsinger, 1976; Kretsinger, 1980; Persechini *et al.*, 1989). The loop contains 7 ligands for the coordination of Ca^{2+} (reviewed in

Strynadka & James, 1989). Five of these ligands are arranged in a pentagonal plane with the other two above and below the plane forming a pentagonal bipyramidal geometry (Strynadka & James, 1989). In this geometry, five amino acid residues contribute 6 oxygen ligands, with a conserved glutamic acid at position 12 in the loop contributing two ligands. The final ligand comes from a water molecule hydrogen bonded to the amino acid residue in position 9. The remainder of the amino acids in the loop stabilize the loop geometry through main-chain NH hydrogen bonding or hydrophobic interactions with other EF-hands (Strynadka & James, 1989).

EF-hand motifs are normally found in pairs (Kretsinger, 1980; Strynadka & James, 1989). These pairs are joined by 5-10 amino acid linker peptides and are related by a 2-fold rotational axis of symmetry. The loops of adjacent EF-hands interact via 2 antiparallel β-sheet hydrogen bonds and hydrophobic interactions. Furthermore, all 4 helices of the EF-hand pair are amphipathic, with their hydrophobic faces packing inwards to form a stabilizing hydrophobic core. In summary, the structural conformation which is responsible for the high affinity Ca^{2+} binding properties of this family of calcium-modulated proteins is stabilized by interactions between EF-hand pairs.

Some Ca^{2+}-modulated proteins are known to undergo a conformational change upon binding Ca^{2+} which leads to interaction with, and activation of target proteins. Perhaps

one of the best characterized Ca^{2+}-modulated proteins is calmodulin. Calmodulin is a small, acidic protein containing four EF-hand Ca^{2+}-binding motifs capable of binding Ca^{2+} with dissociation constants of ~ 10^{-6} μM under conditions of physiological ionic strength and pH for various reviews see (Klee & Vanaman, 1982; Manalan & Klee, 1984; Roberts *et al.*, 1986; Cohen & Klee, 1988). Calmodulin binds target proteins through two hydrophobic clefts which are exposed on the two globular domains of Ca^{2+}-bound calmodulin (Babu *et al.*, 1988; Strynadka & James, 1989; Chattopadhyaya *et al.* 1992). These hydrophobic clefts interact with basic, amphipathic α-helical calmodulin binding domains on the target protein (O'Neil & Degrado, 1990; Ikura *et al.*, 1992; Meador *et al.*, 1992; Meador *et al.*, 1993). The binding of target proteins by calmodulin leads to their activation by relieving the action of their regulatory domain (reviewed in Soderling, 1990). Calmodulin is ubiquitous in eukaryotes, including plants, and it is known to be involved in the Ca^{2+}-dependent mediation of a number of cellular responses (Cheung, 1980; Means *et al.*, 1982; Burger *et al.*, 1984; Manalan & Klee, 1984; Klee & Vanaman, 1982; Cohen & Klee, 1988; Roberts & Harmon, 1992).

Although the presence of calmodulin has been demonstrated in plants, a specific role for this or any other EF-hand protein in plants remains to be thoroughly established (Roberts & Harmon, 1992). Only a few Ca^{2+}/calmodulin-stimulated enzyme activities have been described thus far in

plants. These include Ca^{2+}/calmodulin-dependent NAD kinase (Anderson & Cormier, 1978), a nuclear NTPase (Matsomuto *et al.*, 1984; Chen *et al.*, 1987), the Ca^{2+}-pumping ATPase activity (Dieter & Marme, 1980; 1981; Briars *et al.*, 1988), and a glutamate decarboxylase containing a calmodulin-binding domain (Baum *et al.*, 1993). Recently, pharmacological evidence has suggested the possible existence of a plant homolog of the calmodulin-stimulated phosphatase, calcinuerin (Luan *et al.*, 1993). In addition, a cDNA clone from plants has been isolated that contains a protein kinase catalytic domain consensus sequence and a calmodulin-binding domain (Watillon *et al.*, 1993). The demonstration of Ca^{2+}/calmodulin activation of protein kinase activity for this clone remains to be reported.

All conventional biochemical attempts to demonstrate a calmodulin-dependent protein kinase activity in plants have failed to succeed (reviewed in Roberts & Harmon, 1992). This could be the result of low endogenous levels of this activity or the lack of proper substrates for their identification. However, it is clear that most calcium-dependent protein kinases from plants that have been studied in detail appear to members of the calmodulin-like domain protein kinase family which may be a major Ca^{2+}-target in plant tissues. In the next section the properties of this novel protein kinase class are discussed.

Calmodulin-like domain protein kinase

Recently a novel class of Ca^{2+}-modulated plant protein kinases has been discovered (reviewed in Roberts & Harmon, 1992; Roberts, 1993). It has been established that this class of protein kinases requires neither calmodulin nor phospholipids, and is activated through the direct binding of Ca^{2+} (reviewed in Roberts & Harmon, 1992). In this respect, this family is distinct from previously described Ca^{2+}-dependent protein kinases: Ca^{2+}/calmodulin-dependent protein kinases (reviewed in Edelman *et al.*, 1987; Kennedy *et al.*, 1987; Hanson & Schulman, 1992; Schulman & Lou, 1989) and the protein kinase C family, respectively (reviewed in Nishizuka, 1988; Azzi *et al.*, 1992).

The cDNA sequence of one of these plant Ca^{2+}-dependent protein kinases (Harper *et al.*, 1991) has made it clear why direct activation by calcium is possible, and has clearly established this enzyme as a new class of Ca^{2+}-modulated protein kinase. The sequence of this cDNA clone from soybean contains consensus sequence motifs characteristic of protein kinase catalytic sites (Harper *et al.*, 1991) including the conserved G-X-G-X-X-G-X_n-K nucleotide binding motif and the D-L-K-P-E-N motif associated with protein kinases possessing ser/thr substrate specificities (reviewed in Hanks *et al.*, 1988; Taylor *et al.*, 1992). Overall, the sequence reveals that the molecule contains an amino-terminal catalytic domain (residues 32-295) that is most closely related to the

catalytic domain of the multifunctional calmodulin-dependent protein kinase II (33-42% amino acid identity)(Harper *et al.*, 1991). This catalytic domain is connected via a putative autoinhibitory region to a regulatory domain (residues 329-473) that is 39% identical to spinach calmodulin (Harper *et al.*, 1991). The similarity of this domain to calmodulin includes consensus sequences corresponding to four complete EF-hand Ca^{2+}-binding domains. For this reason, it has been proposed that this class of protein kinases be termed calmodulin-like domain protein kinases (CDPK) (Roberts 1993).

CDPK cDNA clones from *Arabidopsis thaliana* (Harper *et al.*, 1993), carrot (Seun & Choi, 1991), and *Plasmodium faliciparum* (Zhao *et al.*, 1993) have been identified that are similar to the sequence of soybean CDPK. By using monoclonal antibodies against the catalytic domain of the isolated soybean CDPK (Putnam-Evans *et al.*, 1990), crossreactive CDPKs have been detected from a number of sources including oat plasma membrane (Schaller *et al.*, 1992), zucchini plasma membrane (Verhey *et al.*, 1993), *Tradescantia* (Putnam-Evans *et al.*, 1986), and *Chara* (Harmon & McCurdy, 1992). Furthermore, calcium-dependent protein kinases with similar properties have been isolated from *Paramecium* (Gunderson & Nelson, 1987; Son *et al.*, 1993). Also, multiple partially purified kinase preparations with activities similar to purified CDPKs have been described from a variety of plant tissues (reviewed in Roberts & Harmon, 1992). These data indicate that this class

of protein kinase is wide-spread throughout the plant kingdom as well as in some protist species. Finally, the finding of multiple, distinct CDPKs clones from the *Arabidopsis* gene family (Harper *et al.*, 1993), multiple CDPK sequences in soybean (Harper *et al.*, 1991), as well as the apparent existence of both soluble and membrane-associated forms of CDPK (reviewed in Roberts & Harmon, 1992) support the probability that there are multiple CDPK isozymes.

The biochemical properties of several members of this protein kinase family have been described (reviewed in Roberts & Harmon, 1992). Soybean tissue culture CDPK was the first of this family to be purified, and its properties are characteristic of most soluble froms of CDPK described. The enzyme is activated up to 100-fold by μM levels of Ca^{2+}, and it does not require calmodulin nor phosphatidylserine/diacylgycerol effectors for stimulation (Putnam-Evans *et al.*, 1990; Weaver *et al.*, 1991). This property is consistent with the presence of EF-hand motifs within the calmodulin-like domain of the CDPKs (Harper *et al.*, 1991). It has been observed, however, that crude soybean lipids, phosphatidylinositol, and lysophospholipids stimulate the activity of the plasma membrane-associated CDPK from oat up to 20-fold (Schaller *et al.*, 1992; Harper *et al.*, 1993). The stimulation by these lipids is distinguished from the effects of the phospholipid effectors of protein kinase C (Kikkawa *et al.*, 1982) since they do not effect the $K_{0.5}$ of CDPK

for Ca^{2+} (Schaller *et al.*, 1992). The physiological relevance of such stimulation remains to be addressed. In particular it is not clear whether these lipids are bona fide effectors of the kinase or are simply stabilizing the kinase activity *in vitro* (discussed in Roberts, 1993).

Little is known regarding the substrate recognition requirements of CDPKs. Excluding the findings of this study (Weaver & Roberts, 1992), only the H^+-pumping ATPase from oat plasma membrane (Schaller & Sussman, 1988), and the α isoform of the tonoplast intrinsic protein (TIP) (Johnson & Chrispeels, 1992) from bean have been reported as endogenous substrates. Numerous *in vitro* substrates are phosphorylated by CDPKs, including histone H1 (Harmon *et al.*, 1987; Putnam-Evans *et al.*, 1990), casein (Li *et al.*, 1991), BSA (Polya *et al.*, 1987), mylein basic protein (Li *et al.*, 1991), and myosin light chains (Roberts, 1989; Putman-Evans *et al.*, 1990). Synthetic peptide substrates containing either the R/K-X-X-S/T motif (Polya *et al.*, 1989; Roberts, 1989; Putnam-Evans *et al.*, 1990; Weaver *et al.*, 1991) or containing the S-F-K sequence which is found in histone H1 (Olah *et al.*, 1989; Polya *et al.*, 1989) have also been used as *in vitro* CDPK substrates. In contrast, substrates of cAMP-dependent protein kinase which contain the consensus sequence R-R-X-S are generally poor CDPK substrates (Polya *et al.*, 1989; Roberts, 1989; Putnam-Evans *et al.*, 1990).

Excluding the present study, the specific sites of

phosphorylation have only been determined for histone H1 G-A-S-G-S^{PO}4-F-K-L (Olah *et al.*, 1989), α-TIP A-T-R-R-Y-S^{PO}4-F-G-R-T (Johnson & Chrispeels, 1992), and inferred from a myosin light chain derived synthetic peptide K-K-R-P-Q-R-A-T-S^{PO}4-N-V-F-A-M (Roberts, 1989). The sites phosphorylated on these three substrates differ, and at this point it remains unclear what structural characteristics are required for a protein or peptide to be phosphorylated by CDPK.

The identification of CDPKs as a novel class of calcium-dependent protein kinase that is widespread in the plant kingdom, combined with the overall lack of knowledge about mediators and targets of Ca^{2+}-dependent signal transduction in plant cells, has sparked a great deal of research activity in this area. However, because of the fairly recent discovery of this class of Ca^{2+}-modulated enzyme very little progress has been made regarding its role in Ca^{2+}-mediated signal transduction. In the present study, an attempt has been made to address the potential role of this new class of Ca^{2+}-modulated protein kinase within the nitrogen-fixing soybean root nodule.

Nitrogen fixation and nodulation of legumes

The availability of fixed nitrogen in the soil is a critical factor for the survival of plants. It has been recognized for centuries that leguminous plants are able to enrich the quality of the soil (White, 1970). This property is due to a symbiotic relationship between legumes and soil

bacterium of the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* (see Stacey *et al.*, eds., 1992 for a comprehensive review). Through a complex developmental pathway, the bacteria infect the plant leading to the formation of a new organ referred to as a nodule (reviewed in Govers *et al.*, 1987). Inside the nodule conditions are established which allow the bacterium to enzymatically reduce atmospheric nitrogen to form ammonia which is in turn utilized by the plant (Stacey *et al.*, eds., 1992).

Nodule physiology and metabolism

The nodulation of legumes comes about as the result of a complex set of signals sent between the plant and the rhizobia bacterium. The plant roots secrete flavonoid compounds into the soil which induce the transcription of the nodulation (nod) genes of the bacterium (reviewed in Kijne, 1992). It has recently been elucidated that the nodulation genes encode the biosynthetic enzymes which produce an oligosaccharide composed of β-1,4-linked N-acetylglucosamine residues. This structure contains a fatty acid constituent and various modifications such as the addition of sulfate or fucosyl moieties (reviewed in Long *et al.*, 1992; Kijne, 1992). These modifications limit the species of legume that a given bacterium can infect (reviewed in Long *et al.*, 1992). This product, referred to as nod factor is able to induce curling of root hairs and cortical cell division. These processes are necessary for the entrapment of the bacteria and the formation

of the nodule structure, respectively (Faucher *et al.*, 1988; 1989; Kijne, 1992).

The infection/nodulation process has been described in terms of three stages (reviewed in Franssen *et al.*, 1992). First, in the preinfection stage, the bacteria interact with the root hair and induce root hair curling, entrapping the bacteria. Second, during the infection/nodule organogenesis stage, the bacteria invade the plant by breaking down the root hair cell wall. The bacteria enter the root cortex through an infection thread which grows in the direction of the growing nodule primordium in the root cortex. The bacteria are released by endocytosis into the host cell cytoplasm where they become surrounded by a membrane of plant origin, known as the symbiosome membrane (SM)(Roth *et al.*, 1988). This membrane provides the bacterium with protection against plant defense responses since bacterial mutants that have defective SM undergo premature senescence (Werner *et al.*, 1984; 1985). Furthermore, the SM provides a selective barrier that controls metabolic flux between the plant cytosol and the bacterium (reviewed in Day *et al.*, 1990; Day & Udvardi, 1992). Third, in the nodule function/maintenance stage, the bacteria differentiate within the symbiosome to become nitrogen-fixing bacteroids (Fig. 1).

The formation of the nodule structure is accompanied by the enhanced expression of host genes as a result of bacterial infection. These genes are known as nodulins and are proposed

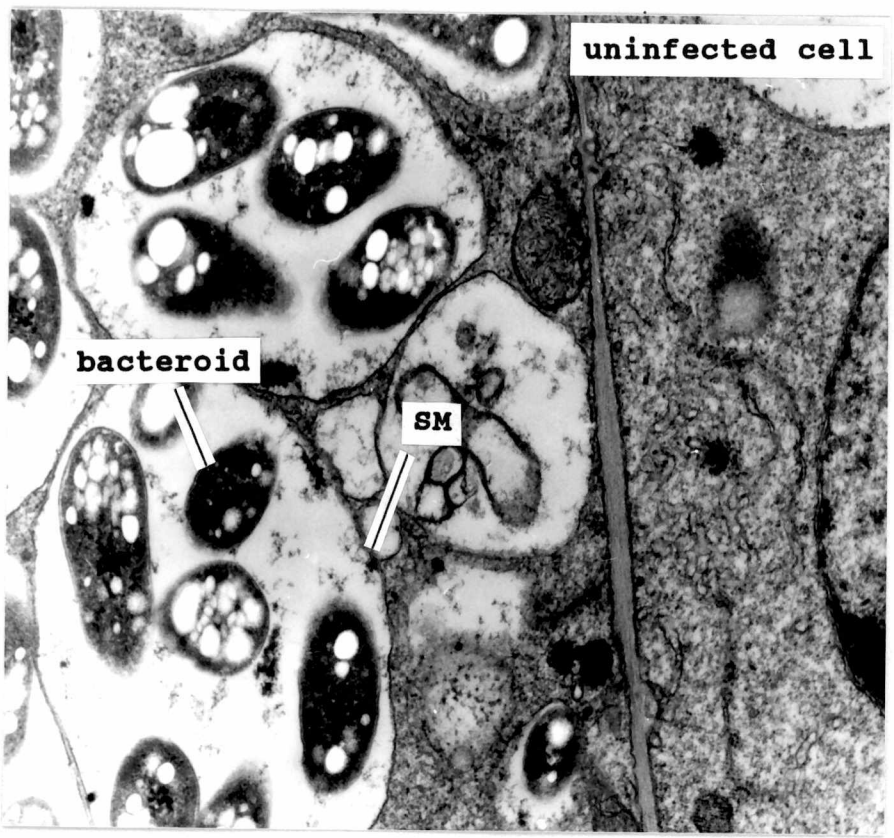

Figure 1. Electron micrograph of infected and uninfected soybean root nodule cells. Electron micrograph of a 28 day post-inoculation nitrogen-fixing soybean root nodule section infected with wild-type *Bradyrhizobium japonicum* strain 61A101C, magnification 20,000 x. Electron micrograph provided by Dr. Evans Roth, Department of Zoology, University of Tennessee.

to encode proteins that play specific roles in supporting the symbiosis (Legocki *et al.*, 1980; Van Kammen *et al.*, 1984). Nodulins are differentially expressed both in terms of spatial location within the nodule structure and the timing of their expression during the nodulation process. Temporal expression is characterized by two broad classes, early and late nodulins. The early nodulins (ENODs) are expressed during the preinfection and infection stages and during the formation of the nodule structure (Franssen *et al.*, 1992). Certain early nodulins, such as GmENOD13, have been implicated in the formation of cell walls of the specialized nodule parenchyma. This specialized cell layer is thought to act as an oxygen barrier necessary for the establishment and maintenance of low oxygen tension that is essential for nitrogen fixation. The physiological functions of other early nodulins is not yet clear.

The other class of nodulins, known as the late nodulins (NODs), are expressed close to the onset of nitrogen fixation (Franssen *et al.*, 1992). Many of this class are "metabolic nodulins" which are involved in carbon, nitrogen, and oxygen metabolism within the nodule. These include the most abundant nodule protein, leghemoglobin (Wittenberg *et al.*, 1974; Sheehy *et al.*, 1985; Govers *et al.*, 1986). This protein comprises 20% of the soluble nodule protein. It acts as a carrier for the facilitated transport of oxygen to the bacteroids in infected cells. This allows the support of respiration while

maintaining low oxygen tension. Other late metabolic nodulins include Ngm-35, a uricase present in uninfected nodule cells (Bergmann *et al.*, 1983) and Ngm-100, a catabolic sucrose synthase (Thummler & Verma, 1987).

Upon the establishment of a nitrogen-fixing symbiosis, a great demand for photosynthate is placed on the host in order to provide energy for the reduction of nitrogen in the bacteroid. The flow of metabolites in the established nodule is shown in Figure 2. Photosynthate (sucrose) transported to the infected cell cytoplasm is converted to glucose-6-phosphate by the catabolic sucrose synthase, Ngm-100. This glucose-6-phosphate is either shunted to the amyloplasts where it is stored as starch, or it is metabolized through glycolysis and eventually to malate via phosphoenolpyruvate carboxylase. Phosphoenolpyruvate carboxylase is a nodule-enhanced enzyme, with levels greater than 50 times those found in the root (Derouche *et al.*, 1983).

Experimental evidence suggests that malate and perhaps other dicarboxylic acids are the primary source of fixed carbon supplied to the bacteroid as an energy source to drive nitrogen fixation (reviewed in Werner, 1992). This idea is supported by the fact that bacterial strains defective in dicarboxylic acid transport (dct) cause the formation of nodules which do not fix nitrogen (fix⁻ nodules) (Finan *et al.*, 1981; Ronson *et al.*, 1981; Bolton *et al.*, 1986). However, although these nodules do not fix nitrogen, they appear

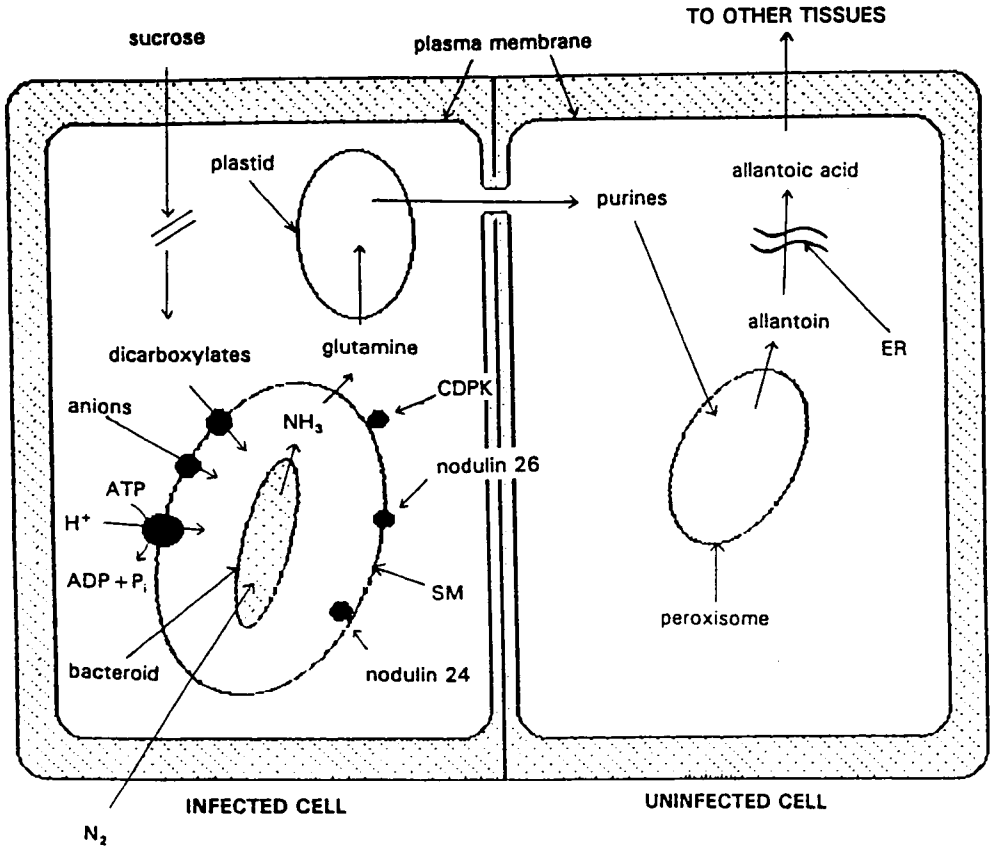

Figure 2. Diagram of infected and uninfected soybean root nodule cells. Based known metabolic pathways in the infected and uninfected nodule cells as reviewed in Werner, 1992. Locations of nodulin 26 and nodulin 24 are based on Fortin *et al.*, 1987, and the present study.

normal, and the bacteria survive within the symbiosomes. This suggests that dicarboxylic acids may not be the carbon source necessary for bacterial survival in the host but that they may be required as fuel to support nitrogen fixation. Furthermore, a promoter regulated by one of the bacteroid's nitrogen-fixation genes, nif A, exists upstream of the dct gene. This suggests that there may be coordinate control of genes involved in nitrogen fixation and the ability of the bacteriods to transport dicarboxylic acids (Ronson & Astwood, 1985).

In exchange for fixed carbon, the bacteroids reduce atmospheric nitrogen to form ammonia. This ammonia is believed to diffuse across the SM by a passive mechanism (Udvardi & Day, 1990) along a concentration gradient established by the production of ammonia by the bacteroid and the rapid removal of cytosolic ammonia by the host glutamine synthetase (Mellor & Werner, 1990)(Fig. 2). The fixed nitrogen in glutamine is then incorporated into purines in the plastids (Mellor & Werner, 1990; Werner, 1992). The purines are exported to the uninfected nodule cells where, in the case of soybean, the purines are converted to allantoin in the peroxisomes by the nodule-specific uricase, Ngm-35 (Mellor & Werner, 1990; Bergmann *et al.*, 1983)(Fig. 2). Conversion of allantoin to allantoic acid in the smooth endoplasmic reticulum provides a form of combined nitrogen that is exported via the xylem to other plant tissues.

The symbiosome and the symbiosome membrane

As mentioned previously, the SM is a membrane of plant origin that surrounds the endosymbiotic bacteroid, separating it from the host cytoplasm. It has been suggested that the SM is responsible for controlling metabolic flux between the plant cytosol and the bacteroid. A number of enzymatic activities and proteins have been described as being associated with the SM (ie., the compartment enclosed within the symbiosome which is external to the bacteroid). Among the activities that have been described are an inward pumping H^+-ATPase capable of generating an electrochemical gradient across the SM (O'Niell *et al.*, 1983; Blumwald *et al.*, 1985; Domingan *et al.*, 1988; Udvardi & Day, 1989; 1991)(Fig. 2). Based on its sensitivity to inhibitors, the SM-ATPase has been proposed to be similar to the plasma membrane type of ATPase that has been previously described (Blumwald *et al.*, 1985). Other activities associated with the SM include a pyrophosphatase (Bassarab & Werner, 1989), a Ca^{2+}-stimulated protein kinase activity (Bassarab & Werner, 1987; Weaver *et al.*, 1991), a dicarboxylic acid transporter (Price *et al.*, 1987; Udvardi *et al.*, 1988; Ou Yang *et al.*, 1990), and a general anion transporter (Udvardi *et al.*, 1989; 1991)(Fig. 2). The proteins that correspond to these activities remain to be purified and identified.

Studies directed at determining the permeability of isolated, intact symbiosomes to various metabolites have

revealed that the SM of soybeans has extremely low permeability to mannitol, glucose, fructose, and sucrose (reviewed in Day *et al.*, 1990; Udvardi *et al.*, 1990; Day & Udvardi, 1993). These compounds appear to cross the membrane by simple passive diffusion based on their slow, unsaturable uptake kinetics (Udvardi *et al.*, 1990). The uptake of amino acids such as glutamate and proline display similar kinetics. The uptake rates for these amino acids are unaffected by energization of the SM by the SM H^+-pumping ATPase (Day *et al.*, 1990).

In contrast to these other compounds, the SM possesses the ability to rapidly transport dicarboxylic acids (Price *et al.*, 1987; Udvardi *et al.*, 1988; Ou Yang *et al.*, 1990; Day & Udvardi, 1993). This transport ability is believed to be the result of a SM dicarboxylic acid transporter (Udvardi *et al.*, 1988). The SM-dicarboxylate transporter exhibits saturation kinetics for malate transport and is competitively inhibited in a concentration-dependent manner by another dicarboxylic acid, succinate (Udvardi *et al.*, 1988; Ou Yang *et al.*, 1990). The apparent K_m for malate is 160 μM and the apparent V_{max} is 10.2 nmol min^{-1} mg $protein^{-1}$ (Ou Yang *et al.*, 1990). The transporter exhibits uptake rates in the presence of submillimolar malate concentrations that are greater than 10-fold in excess of the uptake rates for other substrates, such as succinate (Day *et al.*, 1990). Thus, malate appears to be the preferred substrate for the SM dicarboxylate transporter.

Considering the high malate concentrations estimated for the nodule cytoplasm (~4.5 mM)(Stumpf & Burris, 1979), this rate of uptake is adequate to support nitrogen fixation (Day *et al.*, 1989). This transporter is distinguished from the bacterial dicarboxylic acid transporter by its higher K_m for substrate and its lower affinity for succinate compared to malate (Udvardi *et al.*, 1988). The SM transporter is also inhibited by phthalonate and hydroxycyanocinnamic acid, compounds which do not inhibit the bacteroid dicarboxylate transporter (Udvardi *et al.*, 1988; Day *et al.*, 1990).

Dicarboxylate transport is thought to be driven by the electrochemical gradient established across the SM by the SM-associated H^+-pumping ATPase as well as by a concentration gradient generated by the consumption of malate due to bacteroid respiration (Ou Yang *et al.*, 1990; Day & Udvardi *et al.*, 1992). This model is supported by two lines of evidence. First, treatment with the protonophore CCCP, which destroys the electrochemical gradient across the SM, severely inhibits malate uptake (Ou Yang *et al.*, 1990). These findings suggest that such a gradient is an important driving force for malate transport. Second, the respiratory poison, KCN, which inhibits bacteroid respiration, also inhibits malate uptake without affecting the electrochemical gradient across the SM (Ou Yang *et al.*, 1990).

In addition to these concerns, a third activity associated with the SM may affect dicarboxylate transport. A

general anion transporter, which is driven by the electrochemical gradient established by the H^+-pumping ATPase, has been detected in the SM (Udvardi *et al.*, 1989; 1991). Among the anions transported, nitrate, an effective inhibitor of symbiotic nitrogen fixation, appears to dissipate the gradient most efficiently. In addition to nitrate, malate and a number of other anions are also able to diminish the gradient, albeit with lower efficiency (Udvardi *et al.*, 1989; 1991). Furthermore, similar to the dicarboxylic acid transporter, anion transport is inhibited by phthalonate, an inhibitor of the malate transporter (Ou Yang *et al.*, 1990; Udvardi *et al.*, 1991). Since the general anion transport activity can transport malate and is driven by the trans-SM electrochemical gradient, and since it is inhibited by an inhibitor of the SM-dicarboxylic acid transporter, it appears to be similar to the dicarboxylic acid transporter. However, the specificity of the anion transporter is not as stringent as the dicarboxylate transporter (Day *et al.*, 1990). Thus, much is uncertain with regard to physiologically relevant malate or other anion transport activity. Therefore, more thorough biochemical characterization of the general anion transporter and the dicarboxylate transporter need to be done including the identification of the protein(s) responsible for these activities.

Besides the activities described above, several SM-associated proteins have been identified. Among these are two

nodulins that were cloned from a nodule cDNA library by using cDNA probes generated from mRNA associated with polysomes immunoprecipitated with anti-SM antibodies. One of these proteins is nodulin 24 (Fortin *et al.*, 1987)(Fig. 2). Its function is presently unknown. The other protein is nodulin 26 which is a primary focus of the present study. Comparison of the deduced amino acid sequence of nodulin 26 with other proteins indicates that it belongs to a family of structurally-conserved integral membrane proteins, some of which have been demonstrated to possess transport activities (reviewed in Pao *et al.*, 1991; Reizer *et al.*, 1993). This family is known as the major intrinsic protein (MIP) family based on homology of the member proteins to the MIP of bovine lens. Below a review of the current understanding of the structural and functional properties of the MIP family is presented.

The MIP family

From the most recent reports (Reizer *et al.*, 1993), the MIP family contains eighteen members that share 30-40% amino acid sequence identity with nodulin 26. (Fig. 3) This family, which spans the phylogenetic spectrum, includes the major intrinsic protein (MIP) from vertebrate lens (Gorin *et al.*, 1984), the *Drosophila* neurogenic big brain protein (*bib*)(Rao *et al.*, 1990), pea shoot turgor-induced protein (TUR)(Guerrero *et al.*, 1990), the channel-like intrinsic protein (CHIP) from renal tubules and erythrocytes (Preston & Agre, 1991; Zhang *et*

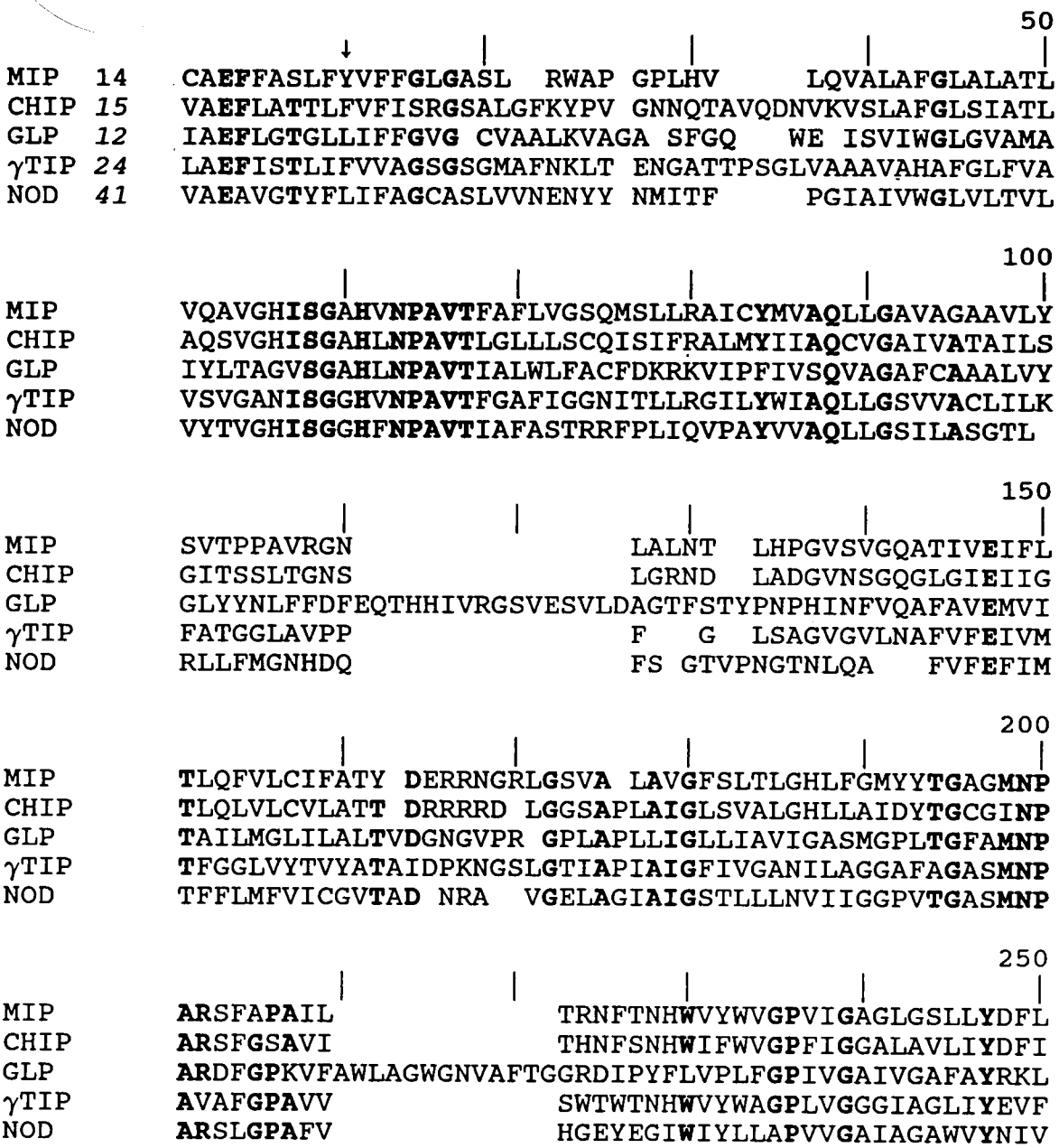

Figure 3. Aligned deduced amino acid sequences of selected members of the MIP family. The sequence alignment is based on the alignment reported in Reizer *et al.* (1993). The numbers in *italics* represent the number of the residue in each of the respective deduced amino acid sequences where the alignment begins. The amino acid residues in **bold** type are amino acids conserved in at least four of the five aligned sequences.

al., 1993; Li *et al.*, 1993), the plant tonoplast intrinsic protein (TIP)(Johnson *et al.*, 1990), yeast (FPS1)(Van Aelst *et al.*, 1991), the glycerol facilitator from *E.coli* (*GlpF*)(Sweet *et al.*, 1990), *Lactococcus lactis* (ORF1)(Mayo *et al.*, 1991), and nodulin 26 (Fortin *et al.*, 1987; Sandal & Marker, 1988; Shiels *et al.*, 1988).

Structural predictions based on hydropathy analysis of the deduced amino acid sequences suggest that the family contains a common structural motif consisting of 6 putative transmembrane spanning α-helices with hydrophilic amino-and carboxyl-terminal domains which are proposed to be exposed to the cytoplasm (reviewed in Reizer *et al.*, 1993)(Fig. 4). Few experimental investigations of the structural properties of members of this family have been undertaken to verify this proposed membrane topology. Orientation of some MIP family members in the membrane has been investigated by limited proteolysis. For example, CHIP is susceptible to carboxypeptidase degradation in inside-out vesicles but not in erythrocytes, suggesting that the free carboxyl terminus is cytosolic (Smith & Agre, 1991). Protease-sensitivity experiments with *in vitro* translation products of nodulin 26 cRNA in the presence of dog pancreas microsomes support an orientation of nodulin 26 with the amino and carboxyl-terminal segments of the protein exposed to the outside of the microsomes (Miao *et al.*, 1992). However, *in vitro* translation and insertion of membrane proteins into microsomal membranes

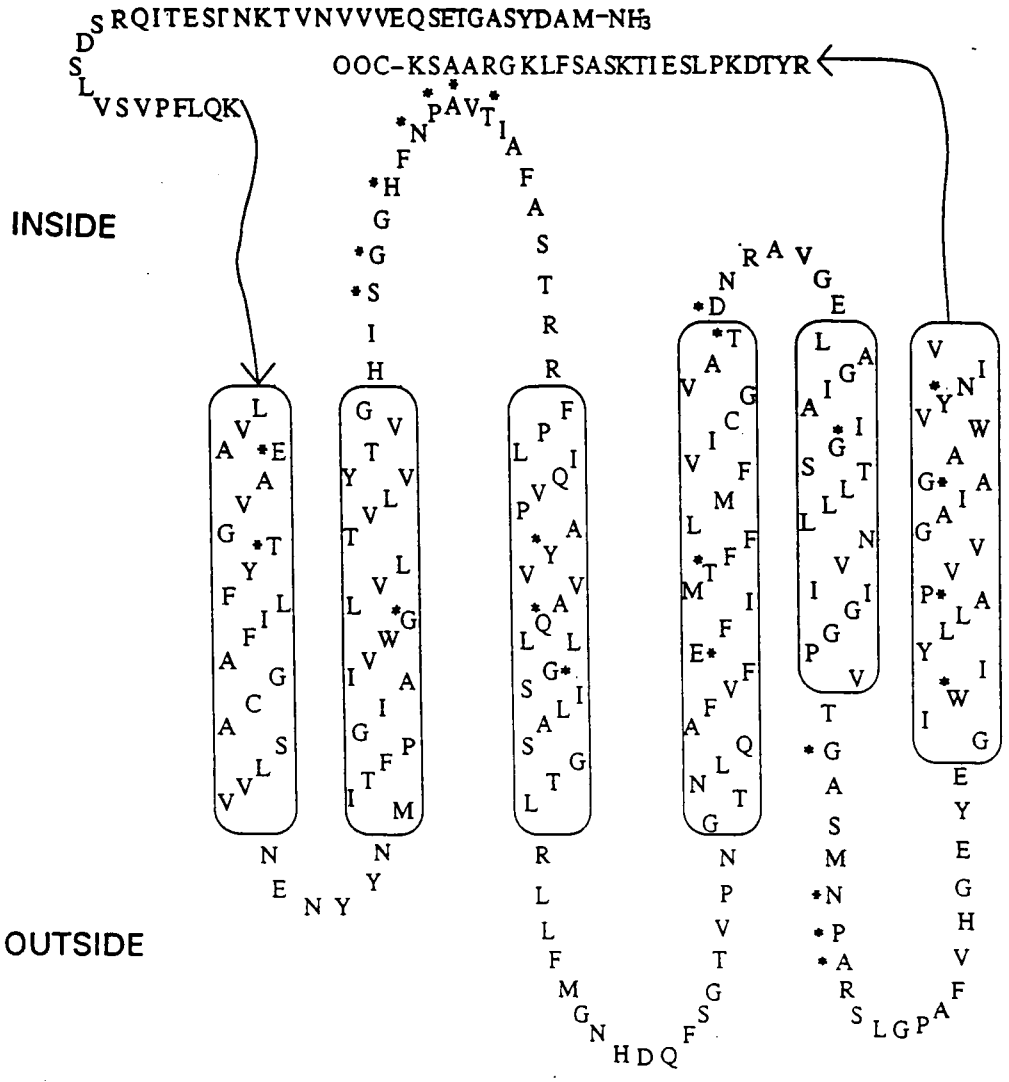

Figure 4. **Proposed membrane topology of nodulin 26.** The proposed topology is based on hydropathy analysis (Kyte & Doolittle, 1982) assuming a cytosolic localization of the COOH- and NH_2-terminal domains based on Miao *et al.*, (1992). Asterisks represent residues that are highly conserved among the MIP family sequences (Reizer *et al.*, 1993).

may not lead to the correct *in vivo* orientation based on a precedent with a gap-junctional protein studied in a similar *in vitro* system (Falk *et al.*, 1993). Despite these caveats, this preliminary study implies a cytosolic localization for the hydrophilic amino- and carboxyl-terminal domains of nodulin 26. Furthermore, the demonstration that MIP is phosphorylated on its carboxyl terminus *in vivo* supports a cytoplasmic orientation for this portion of the molecule (Lampe *et al.*, 1986). Overall, these data suggest that these proteins are oriented with their amino and carboxyl-terminal segments facing the cytoplasm.

Experimental evidence exists that demonstrates that members of this family form tetramers in the membrane. MIP, purified and reconstituted into liposomes as well as in lens fibers, forms tetragonal arrays as observed by freeze fracture electron microscopy (Zampighi *et al.*, 1982; Zampighi *et al.*, 1989; Ehring *et al.*, 1990). Calculation of the volume of the unit cell, and division by the volume of MIP monomer and surrounding lipids, has led to the conclusion that MIP is a tetramer in membranes (Ehring *et al.*, 1990). Freeze fracture electron microscopy of purified CHIP reconstituted into liposomes revealed that CHIP forms similar tetramers when inserted into membranes (Verbavatz *et al.*, 1993). Furthermore, estimation of the molecular mass of detergent solubilized CHIP, by ultracentrifugation, also suggests that solubilized CHIP behaves as a tetramer (Smith & Agre, 1991).

It is not clear at this time, however, how this membrane organization is related to the functional properties of these proteins.

Although many members of the MIP family has been identified, there has been relatively little investigation of the biochemical properties and physiological functions of the proteins in this family. Purified MIP has been studied in planar lipid bilayers. These studies have revealed that MIP appears to form a high conductance, weakly anion-selective, voltage-sensitive ion channel (Ehring *et al.*, 1990). This study supports previous work that indicated that MIP was a channel protein (Gooden *et al.*, 1985; Peracchia & Girsch, 1985; Zampighi *et al.*, 1985) It has been suggested that MIP has a role in maintaining extracellular fluid balance in the lens providing for proper transparency or that it is involved in intercellular communication and transport (Ehring *et al.*, 1990). *GlpF* is able to facilitate the transport of glycerol and other straight chain polyols, but not ionic forms of these molecules, across the plasma membrane of *E. coli* (Heller *et al.*, 1980; Lin *et al.*, 1986). CHIP mediates the specific transport of water across lipid bilayers based on *Xenopus* oocyte expression studies as well as reconstitution studies with the purified protein (Preston *et al.*, 1992; Zeidel *et al.*, 1992; van Hoek and Verkman, 1992). These demonstrations have led to the suggestion that CHIP is responsible for mediating rapid water uptake in erythrocytes and renal tubules

against small concentration gradients. Similar experiments with γ-TIP cRNA expressed in *Xenopus* oocytes demonstrated that it is also a specific water transporter (Maurel *et al.*, 1993). Based on these data and the finding the γ-TIP is specifically expressed in expanding tissues (Ludevid *et al.*, 1992), a role for γ-TIP in turgor maintenance in the vegetative tissues of plants has been proposed. Finally, although no direct identification of its biochemical activity has been done. A transport role for FPS1 has been inferred from yeast complementation studies. Specifically, this protein is able to complement a yeast mutant defective in carbon metabolism possibly by sugar or H^+ transport (Van Aelst *et al.*, 1991).

Although transport roles have been speculated for many of the remaining MIP family members, no direct evidence with regard to their specific transport functions has been offered. Based on the limited structural and functional data available, it seems that the MIP family is composed of a group of proteins that show similarities in structure but possess a diverse range of functional properties. Questions that arise include how are the specific functions of these channels determined by the primary amino acid sequence? With respect to nodulin 26, which of the reported functional properties of this family does it possess, how are these properties regulated, and what role does this protein play in SM-associated transport and nodule physiology?

The purpose of the present study is to identify and

characterize protein kinases capable of detecting and responding to physiologically relevant changes in cytosolic Ca^{2+} concentration in soybean root nodules, and further to attempt to identify targets of these Ca^{2+} sensors in the nodule cells. Emphasis has been placed on determining whether nodulin 26 is a target for Ca^{2+}-dependent phosphorylation, determining the precise location of nodulin 26 in the nodule, determining a function for nodulin 26, investigating how that function may be related to SM transport activity, and finally investigating how Ca^{2+}-dependent phosphorylation may modulate that activity. Success in this endeavor will help clarify the role that Ca^{2+}-dependent phosphorylation plays in plants. Furthermore, studies with nodulin 26 may provide insight into the mechanism of control of metabolic flux and communication between the host and symbiont in the nitrogen-fixing soybean root nodule, and add to the growing body of knowledge of structural and functional relationships between the proteins of the MIP family.

CHAPTER II

MATERIALS AND METHODS

Materials

Endoproteinase Lys-C (Lys-C), trypsin, 1-O-*n*-octyl-β-D-glucopyranoside (OG) and, calf intestinal alkaline phosphatase were purchased from Boehringer-Mannheim. Radionuclear chemicals were purchased from Du Pont-New England Nuclear. Triethylamine, phenylisothiocyanate, ultra-pure Tris, *m*-maleimidobenzoic acid-*N*-hydroxysuccinimide ester (MBS), protein A agarose, and bicinchoninic acid protein assay reagents were purchased from Pierce Chemical Co. Burdick and Jackson brand acetonitrile, heptane, triflouroacetic acid (TFA), and ethyl acetate were purchased from Baxter. High purity formic acid was purchased from Gallard-Schlesinger. Lipids were purchased from Avanti Polar Lipids, and were stored dry at -20° C. DEAE cellulose DE-52, cellulose thin-layer plates, and P81-phosphocellulose paper were purchased from Whatman International. Sephacryl 200 HR, protein A Sepharose and CNBr-activated Sepharose 4B were purchased from Pharmacia. Horseradish peroxidase-coupled goat-anti-rabbit and horse-anti-mouse IgG were purchased from Vector laboratories. Goat-anti-rabbit gold was purchased from Amersham. Goat-anti-rabbit IgG and normal rabbit serum for radioimmunoassay was purchased from Cappel Laboratories. Avid AL affinity chromatography resin was purchased from UniSyn Technologies. C_{18} SEP-PAK cartridges were purchased from

Waters/Millipore. Wicor X-ray film was purchased from Medcore X-ray Systems, Memphis, TN. The CK-15 synthetic peptide was prepared on a Biosearch SAM II automated solid-phase peptide synthesizer by the Molecular Biology Resource Facility at the University of Tennessee, Knoxville. All other reagents were of reagent grade or higher.

Bacterial culture and plant growth

Bradyrhizobium japonicum strains USDA 110 or 61A101C were cultured at 30° C with shaking in Bergenson's minimal medium: $NaH_2PO_4 \cdot 7H_2O$, 270 mg/l; $MgSO_4 \cdot 7H_2O$, 80 mg/l; $FeCl_3 \cdot 6H_2O$, 3 mg/l; ferric monosodium EDTA, 3.7 mg/l; $CaCl_2 \cdot 2H_2O$, 30 mg/l, $MnSO_4 \cdot 4H_2O$, 0.1 mg/l; H_3BO_3, 0.03 mg/l; $ZnSO_4 \cdot 7H_2O$, 0.03 mg/l; $NaMoO_4 \cdot 2H_2O$, 0.0025 mg/l; $CuSO_4 \cdot 5H_2O$, 0.0025 mg/l; $CoCl_2 \cdot 6H_2O$, 0.0025 mg/l; biotin, 0.1 mg/l; thiamine, 1 mg/l; mannitol, 10 g/l; sodium glutamate, 0.5 g/l, yeast extract, 0.5 g/l, pH 7.0. Bacterial cultures were grown until they reached an A_{600} 1.0 (~5 days).

Soybeans seeds (*Glycine max*, cv. Essex) were surface sterilized by the following procedure. Seeds were immersed in 20% (v/v) Clorox for 10 min followed by 5 washes (~500 ml/250 seeds) with sterile H_2O. Seeds were then immersed in 0.01 N HCl for 10 min followed by 5 washes (~500 ml/250 seeds) with sterile H_2O. The sterilized seeds were placed in sterile 150x15 mm glass Petri dishes (~40 seeds/dish) on top of a sheet of Whatman 3 mm Chr paper soaked with ~15 ml of sterile H_2O. The seeds were allowed to germinate for 3 days in the

dark. Germinated seeds were planted in 10 inch pots (5-6 seedlings/pot) containing vermiculite which had been sterilized by autoclaving for 1 hr. Each seedling was inoculated with 1 ml of the bacterial culture described above. The plants were grown in a greenhouse with natural lighting supplemented to 16 h with 400 W multivapor metal-halide lamps. Plants were watered as needed alternately with either tap water or a plant nutrient solution containing: 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; $NaMoO_4 \cdot 2H_2O$, 0.006 mg/l.

Symbiosome membrane purification

SM were purified by a modification of the method of Udvardi & Day (1989). Nodules were harvested, on ice, from soybean plants 28 days after inoculation. The nodules were washed twice on a sieve (~200 ml/wash) with symbiosome wash buffer (20 mM MOPS-NaOH, pH 7.0, 350 mM mannitol, 3 mM $MgSO_4$). Extraction was accomplished by gently crushing nodules with a pestle in a glass mortar containing 20 mM MOPS-NaOH, pH 7.0, 350 mM mannitol, 10 mM EDTA, 10 mM $MgSO_4$, 10 mM DTT, 1% (w/v) PVP-40, 1% (w/v) BSA, 20 mM Na-isoascorbate (1 ml buffer/g nodule fresh weight). The extract was filtered through 1 layer of Miracloth (soaked in extraction buffer) into a glass beaker. The crushed nodule residue was re-extracted in a mortar with a pestle (1 ml buffer/g nodule fresh weight), and

the second extract was filtered through Miracloth and combined with the first extract. The extract was layered, using a 10 ml, wide-mouth (3 mm opening) Pyrex pipet, onto 15 ml step gradients prepared in 30 ml Corex centrifuge tubes. The gradients consisted of equal portions of 30%, 60%, and 80% (v/v) Percoll in 20 mM MOPS-NaOH, pH 7.0, 350 mM mannitol, 3 mM $MgSO_4$. The gradients were centrifuged in a Sorvall HS-4 rotor at 5000 *g* for 15 min at 4° C, and were allowed to decelerate without braking. The gradients were harvested by aspiration of the soluble extract (remaining on top of the Percoll) and the 30% Percoll fraction. The 60% Percoll fraction was then carefully removed down to the position of the band of intact symbiosomes at the 60-80% Percoll interface. This band was harvested by gentle pipetting with a 10 ml, wide-mouth (3 mm opening) Pyrex pipet. The intact symbiosomes were dispersed in symbiosome wash buffer (5 ml/g nodule fresh weight) in 150 ml Corex centrifuge tubes and were centrifuged at 650 *g* for 3.5 min at 4° C in a Sorvall GSA-3 rotor. The turbid supernatant fraction was aspirated and the symbiosome pellet was resuspended in 20 mM MOPS-NaOH, pH 7.0, 150 mM KCl (0.2 ml buffer/g nodule fresh weight). The symbiosomes were ruptured by extruding them twice through a 25 gauge x 0.5 inch hypodermic needle connected to a syringe. The ruptured symbiosomes were centrifuged in a 30 ml Corex centrifuge tube at 6000 *g* for 10 min at 4° C in a Sorvall HS-4 rotor. The supernatant fraction was decanted and saved. The

pellet was resuspended in 20 mM MOPS, pH 7.0, 150 mM KCl, and was extruded twice through a 25 gauge x 0.5 inch needle. The sample was centrifuged as described above. The supernatant fraction was decanted and combined with the first supernatant fraction. The combined fractions were centrifuged at 100,000 *g* for 1 h at 4° C in a Beckman Ti 60 rotor. The supernatant fraction was discarded and the pellet, which contained purified SM, was resuspended in 20 mM MOPS-NaOH, pH 7.0, 0.5 mM EGTA and, unless stated differently, was stored at -80° C.

Protein kinase assays

Protein kinase assays were performed by the phosphocellulose filter assay method described by Roberts *et al*. (1984) with modifications. CDPK activity was detected by the incorporation of ^{32}P into the CK-15 synthetic peptide. Typical reaction mixtures (50 μl total volume) contained 129 μM CK-15, 25 mM MOPS-NaOH, pH 7.0, 10 mM magnesium acetate, 1 mM DTT, 0.4 mM γ-^{32}P-ATP (500 cpm/pmol), 0.5 mM $CaCl_2$, and varying amounts of CDPK-containing samples. 1 mM EGTA was substituted for $CaCl_2$ to establish Ca^{2+} dependency. Reactions were initiated by the addition of enzyme and incubated for 10-30 min at 30°C. Reactions were terminated by spotting 15-25 μl of the reaction mixture onto 1 cm squares of phosphocellulose paper. The squares were allowed to dry for 2 min and were then washed 4 times in 75 mM H_3PO_4 (10 ml/square, 10 min/wash). The squares were washed once in 95% ethanol and dried. The papers were suspended in 10 ml of

BetaMax liquid scintillation cocktail and radioactivity was quantitated using a Beckman 3081 liquid scintillation counter.

For determination of the $K_{0.5}$ for Ca^{2+}-stimulation, the Ca^{2+} concentration was varied over a range of 7.2×10^{-9}-1.4×10^{-4} M. A computer program (Perrin & Sayce, 1967) and the stability constants of Martell & Smith (1974) were used to determine the amounts of $CaCl_2$ and EDTA necessary to yield the desired free Ca^{2+} concentrations. All buffers and the CK-15 substrate were treated with Chelex resin prior to use to remove contaminating metal ions. The metal ion content of the treated solutions was found to be negligible based on atomic absorption spectroscopy analysis performed using an Instrumentation Laboratories model 551 atomic absorption spectrometer. The dependence of the soluble CDPK on exogenous calmodulin was determined by comparing the activity of the Mono Q-purified CDPK in assays performed in the presence or absence of 100 nM spinach calmodulin. The dependence of the soluble CDPK on phosphatidylserine and diacylglycerol was determined by comparing the activity in the presence and absence of liposomes containing 24 μM phosphatidylserine and 0.64 μM 1,2-dioleylglycerol as described in Kikkawa *et al.* (1982). The effect of ionic strength on the activity of soluble CDPK was determined by performing protein kinase assays under standard conditions and varying the concentration of KCl from 0-0.5 M.

Soluble CDPK purification

Soybean root nodules (from plants 28 days after inoculation) were harvested and frozen in liquid nitrogen. Nodules were thawed and ground with a pestle in a mortar containing ice-cold buffer 25 mM MOPS-NaOH, pH 7.2, 5 mM EGTA, 1% (w/v) PVPP, 10% (v/v) glycerol, 5 mM DTT, 20 mM Na-isoascorbate, 5 μg/ml leupeptin, 3 μg/ml pepstatin, 90 μg/ml TPCK, 90 μg/ml PMSF, and 1 μg/ml soybean trypsin inhibitor (3 ml buffer/g nodule fresh weight). The extract was filtered through Miracloth and was centrifuged at 100,000 *g* for 30 min at 4° C in a Beckman Ti 60 rotor. The supernatant fraction was brought to a concentration of 8% (w/v) PEG 8000 by the addition of a 50% PEG 8000 stock solution. The sample was stirred on ice for 30 min and was centrifuged at 14,000 *g* for 30 min at 4° C in a Sorvall GS-3 rotor. The supernatant fraction was adjusted to 28% (w/v) PEG 8000 and was stirred and centrifuged as described above. The supernatant fraction was discarded and the pellets were resuspended in DEAE-chromatography buffer (20 mM MOPS-NaOH, pH 7.0, 1 mM EGTA, 5 mM β-ME, 0.1 M NaCl, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin, 50 μg/ml TPCK, and 50 μg/ml PMSF). The resuspended sample was clarified by centrifugation at 35,000 *g* for 5 min at 4° C in a Sorvall SS-34 rotor. The supernatant fraction was applied to a DEAE-cellulose DE-52 column (1 ml resin/g nodule fresh weight) equilibrated in DEAE-chromatography buffer. The column was washed until the A_{280} of the effluent was zero. The

column was eluted with a linear gradient of 0.1-0.4 M NaCl in DEAE-chromatography buffer. Fractions containing CK-15 kinase activity were pooled and concentrated by ultrafiltration in an Amicon stirred cell with a YM-30 membrane. The concentrated sample (~20 ml) was applied to a 2.6 x 100 cm Sephacryl HR 200 column. The column was eluted with 20 mM MOPS-NaOH, pH 7.0, 0.5 mM EGTA, 5 mM β-ME, 0.2 M NaCl, 0.5 μg/ml leupeptin, 0.3 μg/ml pepstatin, 50 μg/ml TPCK, and 50 μg/ml PMSF at a flow rate of 15 ml/h. Fractions with protein kinase activity were pooled and concentrated by ultrafiltration as described above. Approximately two volumes of 25 mM Tris-HCl, pH 7.4, 1 mM $CaCl_2$, 0.1 mM DTT was added to the concentrated sample, and it was applied to a Mono Q 5/5 column, attached to a Pharmacia FPLC. The column was equilibrated in 25 mM Tris-HCl, pH 7.4, 0.5 mM $CaCl_2$, 0.1 mM DTT, 0.1 M NaCl, and 0.5 μg/ml leupeptin (Mono Q-chromatography buffer). The column was washed until the A_{280} reached baseline and was then eluted with a linear gradient of 0.1-0.4 M NaCl in Mono Q-chromatography buffer. Fractions containing protein kinase activity were pooled and concentrated by dialysis against 25 mM Tris-HCl, pH 7.4, 0.1 mM EGTA, 0.1 mM DTT, 50% sucrose, and 0.5 μg/ml leupeptin at 4° C. The concentrated kinase was stored at -80° C.

Partial purification of the SM-associated CDPK

Purified, pelleted SM were rinsed in 10 mM sodium phosphate, pH 7.5, and were resuspended in 10 ml of 7.5 mM sodium phosphate, pH 7.5, 1 M KI, 1 mM EDTA, 1 mM DTT, 1 mM

PMSF, 1 μM leupeptin, 1 μM pepstatin. The sample was incubated for 30 min at room temperature, and was centrifuged at 100,000 *g* for 1 h at 4° C in a Beckman Ti 60 rotor. The supernatant fraction was discarded. The pellet was rinsed with 20 mM MOPS-NaOH, pH 7.0 and was resuspended in 20 mM Tris-HCl, pH 7.0, 1 mM EDTA, 2% (w/v) CHAPS, 1 mM PMSF, 1 μM leupeptin, and 1 μM pepstatin (~2 ml/mg SM protein). Following a 2 h incubation on ice, the sample was centrifuged at 100,000 *g* for 1 h at 4° C in a Beckman Ti 60 rotor. The supernatant fraction was applied at a flow rate of 0.5 ml/min to a Mono Q 5/5 column attached to a FPLC. The column was washed with 20 mM Tris-HCl, pH 7.0, 0.5 mM EGTA, and 0.5% (w/v) CHAPS until the A_{280} reached baseline. The column was eluted with a linear gradient of 0-0.5 M NaCl in 20 mM Tris-HCl, pH 7.0, 0.5 mM EGTA, 0.5% (w/v) CHAPS. Fractions (0.5 ml) were collected and were assayed for protein kinase activity under standard conditions.

Phosphorylation of nodulin 26 by the SM-associated CDPK

For *in vitro* phosphorylation of nodulin 26, isolated SM were incubated in a reaction mixture containing 0.4 mM γ-^{32}P-ATP (0.45 μCi/nmol), 0.5 mM $CaCl_2$, 25 mM MOPS-NaOH, pH 7.0, 10 mM magnesium acetate, and 1 mM DTT for 20 min at 30° C. The labeled membranes were assayed directly by SDS-PAGE. For phosphorylation site determination, labeled SM were centrifuged at 100,000 *g* for 1 h at 4° C in a Beckman Ti 50

rotor to remove residual γ-^{32}P-ATP. The pellet was rinsed twice with 50 mM Tris-HCl, pH 7.8 prior to analysis.

^{32}P-labeling of nodulin 26 in intact soybean nodules

Soybean root nodules were immersed in 0.85 mg/l KH_2PO_4, 1.09 mg/l K_2HPO_4, 18.7 mg/l KCl, 123.3 mg/l $MgSO_4 \cdot 7\ H_2O$, 27.7 mg/l $CaCl_2$, 8.7 mg/l ferric monosodium EDTA (1 ml buffer/g nodules fresh weight). ^{32}P-phosphate (0.5 mCi/g nodule fresh weight) was added and the nodules were incubated for 2 h at room temperature with shaking. The nodules were extracted and SM were isolated as described above except for the substitution of 10 mM EDTA for 10 mM EGTA and the addition of 10 mM NaF and 10 mM sodium pyrophosphate (phosphatase inhibitors) to the extraction buffer. $^{32}PO_4$ incorporation into proteins was detected by autoradiography after SDS-PAGE.

Phosphoamino acid and phosphorylation site analyses

CK-15 (200 μg) was phosphorylated by the SM-associated CDPK in a reaction mixture containing SM (475 μg protein), 0.4 mM γ-^{32}P-ATP (0.45 μCi/nmol), 0.5 mM $CaCl_2$, 25 mM MOPS-NaOH, pH 7.0, 10 mM magnesium acetate, and 1 mM DTT for 20 min at 30° C. CK-15 was purified from the other components of the reaction mixture by chromatography on a C_{18} SEP-PAK cartridge. The reaction mixture was applied to the cartridge, and then were washed with 3 ml of 0.1% (v/v) TFA in water. The column was eluted in a stepwise fashion with 3 ml of 0.1% (v/v) TFA, 10% (v/v) acetonitrile followed by 3 ml of 0.1% (v/v) TFA, 32% (v/v) acetonitrile. All fractions (0.5 ml/fraction) were

analyzed for phospho-CK-15 by the phosphocellulose filter assay method described above. Phospho-CK-15 was detected in the 0.1% (v/v) TFA, 32% (v/v) acetonitrile eluate. These fractions were pooled, frozen, and lyophilized.

Nodulin 26 was labeled *in vitro* in SM or *in vivo* in intact nodules as described above. After collection of SM by centrifugation, nodulin 26 was hydrolyzed in 6 N HCL for 2 h at 105° C. Hydrolyzed nodulin 26 was dried under vacuum and analyzed for phosphoserine and phosphothreonine by applying the samples, along with 0.4 μg phosphoserine and phosphothreonine standards, onto cellulose thin-layer plates. Thin-layer electrophoresis (TLE) was performed at 900 V for 2 h in 7.8% (v/v) acetic acid, 2.5% formic acid, pH 2.0 (Roberts, 1989). Amino acid standards were visualized by spraying the plate with a ninhydrin solution (0.2% [w/v] in n-butanol) followed by incubation for 10 min at 100° C. ^{32}P-labeled amino acid were visualized by autoradiography.

Proteolytic digestion of phosphorylated nodulin 26 was performed by resuspending labeled and washed SM in 50 mM Tris-HCl, pH 7.8, 0.1% (w/v) SDS to yield a protein concentration of 1 mg/ml. The sample was heated at 85° C for 5 min and was centrifuged at 100,000 *g* for 1 h at 25° C in a Beckman Ti 50 rotor. The supernatant fraction was decanted and saved. Lys-C (1:100 [w/w] protease/SM protein) or trypsin (1:50 [w/w] protease/SM protein) was added to the solubilized SM and the samples were incubated overnight at 37° C. Proteolytic

digestion of labeled CK-15 was performed by incubating purified phospho-CK-15 with Lys-C (1:50 (w/w) protease/peptide) in 50 mM Tris-HCl, pH 7.8 overnight at 37° C.

Peptides were isolated from other components of the proteolytic digests by chromatography on C_{18} SEP-PAK cartridges. The samples were applied to the cartridges and were washed with 3 ml 0.1% (v/v) TFA in water. Peptides were eluted in a stepwise fashion with 30%, 50%, and 90% (v/v) acetonitrile in 0.1% (v/v) TFA (3 ml/step). The eluate was monitored for phosphopeptides by measuring Cerenkov radiation. All eluted phosphopeptides from digests of SM and CK-15 were recovered in the 30% (v/v) acetonitrile, 0.1% (v/v) TFA fractions. The SM and CK-15 phosphopeptide fractions were pooled separately, frozen, and lyophilized.

Phosphopeptide mapping was performed by TLE and thin-layer chromatography (TLC) analyses of C_{18} SEP-PAK-purified phosphopeptides. TLE was performed by applying phosphopeptides to cellulose thin-layer plates along with ^{32}P-phosphate and γ-^{32}P-ATP as standards. Electrophoresis was performed on a Pharmacia flatbed electrophoresis apparatus in formic acid/acetic acid/water, 5:15:80 (v/v), at 1000 V for 40 min as described by Adams *et al.* (1989). TLC was performed by applying phosphopeptide samples to cellulose thin-layer plates as described above. Plates were incubated in an atmosphere saturated with TLC buffer (butanol/pyridine/acetic acid/water,

45:30:9:36 [v/v]) for 30 min. Ascending chromatography was performed in TLC buffer as described by King *et al.* (1983). Phosphopeptides were detected by autoradiography.

Two dimensional analysis by TLE and TLC was performed by mixing Lys-C digested phospho-CK-15 and nodulin 26 phosphopeptides. Mixed samples were applied to cellulose thin-layer plates, and TLE was performed as described above. The plate was allowed to dry completely, and TLC was performed as described above. Phosphopeptides were detected by autoradiography.

HPLC chromatography was performed by resuspending C_{18} SEP-PAK purified ^{32}P-phosphopeptides in 200 μl of 0.06% (v/v) TFA in water. The samples were chromatographed on a Vydac C_{18} column (0.46 x 25 cm) by using a Waters HPLC. Chromatography buffers were 0.06% (v/v) TFA in water (buffer A) and 0.052% (v/v) TFA, 80% (v/v) acetonitrile (buffer B). Phosphopeptides were eluted with a 12.5-43% linear gradient of buffer B at a flow rate of 0.5 ml/min. 100 μl fractions were collected. Phosphopeptide elution was monitored by measuring Cerenkov radiation, and peptide elution was monitored by absorbance at 210 nm.

Manual Edman degradation was performed essentially as described by Tarr (1986) and Girault *et al.* (1989). Purified phosphopeptides were placed into five 6x15 mm borosilicate glass tubes (2000 cpm/tube) and dried under vacuum. Each cycle of Edman degradation consisted of the following steps:

addition of 5 μl of triethylamine, drying under vacuum, addition of 10 μl of 95% ethanol/triethylamine/phenyl isothiocynate, 7:2:1 (v/v), incubation for 15 min at 50° C, drying under vacuum, two washes with 200 μl of heptane/ethylacetate 17:1 (v/v), and drying under vacuum. Cleavage was accomplished by the addition of 20 μl of TFA and incubation for 10 min at 50° C. Following cleavage the samples were dried under vacuum. At the completion of each cycle, one tube for each peptide was withdrawn from the process. Radioactive products were eluted from the sequencing tubes with 100 μl of acetic acid/formic acid/water, 8:2:90 (v/v). The eluates were dried under vacuum and resuspended in 8 μl of acetic acid/formic acid/water 8:2:90 (v/v). The samples were applied to cellulose thin-layer plates, along with ^{32}P-phosphate as a standard, and were subjected to TLE in formic acid/acetic acid/water 5:15:80 (v/v) for 2 h at 100 V. After electrophoresis, the plates were dried, and radioactive products were detected by autoradiography.

Nondenaturing purification of nodulin 26

Isolated SM (1 mg protein) were rinsed with 10 mM sodium phosphate, pH 7.5, and were resuspended in 10 ml of 7.5 mM sodium phosphate, pH 7.5, 1 M KI, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 μM leupeptin, 1 μM pepstatin. The sample was incubated for 30 min at room temperature, and was centrifuged at 100,000 *g* for 1 h at 4° C in a Beckman Ti 60 rotor. The supernatant fraction was discarded and the pellet was rinsed

twice with 5 ml of 10 mM sodium phosphate, pH 7.9. The pellet was resuspended in 1 ml of 50 mM sodium phosphate, pH 7.9, 2% (w/v) OG, 1 mM NaN_3, 1 mM PMSF, 1 μM leupeptin, 1 μM pepstatin, and was incubated overnight at 4° C. The sample was centrifuged at 100,000 *g* for 1 h at 4° C in a Beckman Ti 60 rotor. The supernatant fraction was applied at a flow rate of 0.25 ml/min to a 1.8 ml DEAE Fast Flow column attached to a Pharmacia FPLC. The column was washed with 10 column volumes of 10 mM Tris-HCl, pH 8.0, 1% (w/v) OG at a flow rate of 0.5 ml/min and then with 10 column volumes of 20 mM MES-NaOH, pH 6.2, 1% (w/v) OG. The column was eluted with a 30 ml linear gradient of 0-0.4 M NaCl in 20 mM MES-NaOH, pH 6.2, 1% (w/v) OG. Fractions (0.5 ml) were collected and were analyzed by SDS-PAGE and by Western blots with anti-CK-15 antibodies. Fractions containing purified nodulin 26 (100 μg) were pooled and concentrated to 0.2 mg/ml using a Centricon-10 ultrafiltration device.

Electrophysiology methods

Nodulin 26 was reconstituted into liposomes by the method of Ehring *et al.* (1990). Briefly, 100 μg of purified nodulin 26 was added to 3.6 μmol soybean phosphatidylcholine in 470 mM OG, 200 mM NaCl, 4 mM Tris base, 5 mM EDTA, 1 mM $CaCl_2$, 1.5 mM NaN_3 (total volume, 714 μl). The sample was dialyzed against 3 l of 25 mM TES-NaOH, pH 7.0, 200 mM NaCl in a dialysis bag with a M_r cutoff of 3,500. The buffer was changed every 24 h over a 4 day period. Nodulin 26-free liposomes were made by

the same procedure except that 20 mM MES-NaOH, pH 6.2, 1% (w/v) OG, 200 mM NaCl was substituted in place of purified nodulin 26.

Nodulin 26 ion channel activity was investigated by fusion of nodulin 26 proteoliposomes with planar lipid bilayers. Meuller-Rudin planar lipid bilayers were formed by painting a 50 mg/ml mixture of bovine brain phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine, (5:3:2 ratio by weight) dissolved in n-decane, across a 250 μm aperture in a Delrin cup. For standard recording conditions, both compartments of the bilayer chamber were filled with 1 M KCl, 20 mM MOPS-NaOH, pH 7.4 (free Ca^{2+} 7 μM) in a *cis* volume of 0.5 ml and a *trans* volume of 3.0 ml. For experiments with asymmetric buffer conditions, the solution in the *trans* chamber was removed and replaced with the appropriate buffer, as indicated. BSA (3 mg/ml) was added to each chamber prior to liposome addition. The addition of BSA alone had no effect on the properties of the bilayer. 100 μl of nodulin 26 proteoliposomes were added to 5 μl of a 5% (w/v) bovine brain phosphatidylcholine/10% (w/v) CHAPS mixture. 10 μl of this mixture were added to the *cis* and *trans* chambers. Additional nodulin 26 proteoliposomes (10 μl) were added at 15 min intervals until channel incorporation was observed.

A custom-designed headstage and amplifier (Hamilton *et al.*, 1989) were connected to the *cis* chamber of the bilayer

apparatus via a Ag-AgCl electrode and an agar bridge, while the *trans* chamber was held at virtual ground. Analog data was filtered at 2500 Hz through an 8-pole Bessel filter (Frequency Devices model 902), digitized (TL1 DMA interface, Axon Instruments), and stored on a Bernoulli T90 disk (IOMEGA). Data was collected using either a pulsing or a ramp protocol. For the pulsing protocol, the potential was held at 0 mV for 0-4 sec and was pulsed for 1-2 sec to the test potential. For reversal potential determinations, a ramp protocol was used in which the voltage was increased from -100 mV to +100 mV at a rate of 0.1 mV/msec (CLAMPEX program, PCLAMP software, Axon Instruments). Current amplitudes are expressed as absolute deviations from zero because the possibility of channel protein incorporation from either side of the bilayer precludes the identification of the sidedness of the channel. Single channel current amplitudes and conductance state percent occupancy times were analyzed with FETCHAN and PSTAT programs (PCLAMP software, Axon Instruments). The TRANSIT program (Van Dongen, 1992) was used to generate conductance amplitude histograms. The permeability ratio PK^+:PCl^- was calculated from a derivation of the Goldman-Hodgkin equation:

$$E_r = \frac{RT \ln ([\text{cation trans}] + PK^+/PCl^- [\text{anion cis}])}{Fz ([\text{cation cis}] + PK^+/PCl^- [\text{anion trans}])}$$

where E_r = reversal potential, R = gas constant,

T = temperature, F = Faraday's constant, z = valence

Immunochemical methods

Nodulin 26 antisera were produced for this study by using synthetic peptides and gel purified nodulin 26 as antigen. For production of nodulin 26 site-directed antibodies, the synthetic peptide CK-15 was conjugated to ovalbumin using MBS according to the protocol of Doolittle (1986). Briefly, 1ml of 20 mg/ml ovalbumin in 0.01 M sodium phosphate, pH 7.0 was combined with 100 μl of 25 mg/ml MBS in dimethylformamide. This mixture was incubated for 30 min at room temperature. Following the incubation, excess MBS was removed by gel filtration on a 1.5x20 cm Sephadex G-10 column. Fractions containing protein were detected by absorbance at 280 nm. Ovalbumin-MBS conjugate (2 ml) was mixed with 200 μl of ^{32}P-CK-15 (1:100 ratio of phospho-CK-15:CK-15; 300,000 cpm total) in 0.1 M sodium phosphate, pH 6.5, 2 mM EDTA. This mixture was incubated for 4 h at room temperature. After the incubation, the ovalbumin-CK-15 conjugate was dialyzed against 4 l of 1 mM n-ethylmaleimide overnight at 4° C, followed by dialysis against 4 l of PBS overnight at 4° C. Conjugation efficiency was determined by measuring the radioactivity in the sample after dialysis by liquid scintillation counting. Purified nodulin 26 for antibody production was prepared by the solubilization of purified SM (~1 mg protein) in SDS-PAGE sample buffer. Nodulin 26 was purified from solubilized SM by SDS-PAGE in a Bio-Rad Model 491 Prep Cell.

New Zealand white rabbits were injected subcutaneously with 100 μg of antigen emulsified in 1 ml of PBS and Freund's complete adjuvant (1:1). After 1 week the rabbits were injected with the same amount of antigen emulsified in 1 ml of PBS and Freund's incomplete adjuvant (1:1). After an additional week, the rabbits were injected a third time with the same amount of antigen in 1 ml of PBS. Rabbits were bled at weekly intervals after the third injection. Production of antibodies was monitored by Western blotting using purified SM as a source of antigen and by ELISA using the unconjugated CK-15 or purified SM as antigen.

Purification of IgG fractions was performed on protein A Sepharose or protein A agarose. Crude serum was diluted 1:10 in 20 mM Tris-HCl, pH 7.5, 0.15 M KCl (loading buffer) and was applied (0.5 ml/min) to the protein A column equilibrated in loading buffer. The column was washed with 30 column volumes of wash buffer and was eluted with 5 column volumes of 200 mM glycine, pH 2.5. Alternatively, IgG was purified on Avid AL resin by the manufacturer's (UniSyn Technologies) protocol. Fractions were collected in tubes containing 0.1 vol. of 1 M Tris base. Protein was detected in the eluate by monitoring A_{280}. Fractions containing IgG were pooled and were dialyzed against 4 l of PBS at 4° C.

Affinity purification of the anti CK-15 antibody was done by chromatography on a CK-15 column. This column was prepared by first immobilizing BSA on CNBr activated Sepharose-4B by

the manufacturer's protocol. CK-15 was conjugated to the BSA Sepharose with MBS as described by Doolittle (1986) for the conjugation of peptides to carrier proteins. Chromatography of anti-CK-15 serum on the resin was performed by the general affinity chromatography approach described by Harlow & Lane (1988). Purified antibody fractions were dialyzed against 4 l of PBS at 4° C and were concentrated by ultrafiltration using an Amicon stirred cell with a YM-30 membrane or by using a Centricon 30 ultrafiltration device. Purified antibodies were stored at -80° C until use.

Western blots were done by electrophoretic transfer of proteins from SDS-PAGE gels to nitrocellulose filters (100 mA constant current overnight in a Bio-Rad Trans-blot Cell or 1 A constant current for 1 h in an Idea Scientific Genie Blotter). Filters were blocked in 150x15 mm plastic Petri dishes with 10% (w/v) nonfat dry milk in PBS. Primary antibody incubations were done in PBS, 0.5% (v/v) normal goat serum for 1 h at room temperature with the following antibody dilutions: anti-CK-15 IgG, 6 μg/ml; anti-nodulin 26 IgG, 9 μg/ml. The filters were washed three times (30 ml/wash) with PBS, 0.05% (v/v) Tween 20 for 10 min/wash. Secondary antibody incubations were performed at room temperature with 0.75 μg/ml horseradish peroxidase-coupled goat-anti-rabbit antibody in PBS, 0.05% (v/v) Tween 20, 0.5% (v/v) normal goat serum for 1 h. The filters were washed three times (30 ml/wash) with PBS, 0.05% (v/v) Tween 20 for 10 min/wash. The filters were

developed with 0.5 mg/ml 4-chloro-1-naphthol, and 0.01% (v/v) H_2O_2 in PBS. As a negative control, preimmunization serum was substituted for antisera in the above protocol.

For detection of CDPK, a modified Western blot protocol was employed. Anti-CDPK monoclonal antibodies (Putnam Evans *et al.* 1990) were combined and diluted (12G8, 5 μg/ml; 3E8, 15 μg/ml; 3C11, 10 μg/ml; 2E1, 22 μg/ml) in PBS. Horseradish peroxidase-coupled horse-anti-mouse IgG was used instead of goat-anti-rabbit IgG conjugate. Incubations times, washes, and the developing protocol remained the same as above.

ELISA were performed to determine anti-CK-15 antibody titers by adsorbing 50 μl of 20 μg/ml CK-15 in 50 mM sodium carbonate, pH 9.5 to polystyrene microtiter plates overnight at 4° C. The solution was aspirated and the plates were blocked for 2 h with 2 mg/ml BSA in 200 mM glycine, pH 7.0 (200 μl/well). The blocking solution was aspirated and the plates were washed 5 times with PBS. Varying dilutions of primary antibodies (50 μl/well) were prepared in PBS, 0.5% (v/v) normal goat serum and incubated for 1 h at room temperature. The solution was aspirated and the plates were washed 5 times with PBS. Horseradish peroxidase-coupled goat-anti-rabbit antibody (0.2 μg/ml) in PBS, 0.5% (v/v) normal goat serum was added (50 μl/well) and the plates were incubated for 1 h at room temperature. The solution was aspirated and the plates were washed 5 times with PBS. Bio-Rad peroxidase substrate solutions were added (50 μl/well) and

incubated 30 min in the dark. The product was detected at 540 nm with a Bio-Tek EL310 microplate reader.

Comparison of nodulin 26 protein levels in wild-type and fix⁻-nodules was performed on nodules arising from the infection of soybean by wild type 61A101C and mutant ML126, ML146, and ML150 *Bradyrhizobium japonicum*. The mutant strains were generated by Tn5 insertional mutagenesis as previously described in So *et al*. (1987). Nodules were frozen in liquid nitrogen and ground in a mortar with a pestle. The ground nodules were thawed in extraction buffer (25 mM MOPS-NaOH, pH 7.0, 10 mM EGTA, 10 mM $MgSO_4$, 5 mM Na-isoascorbate, 5 mM DTT, and 1% [w/v] PVP-40) (2 ml buffer/g nodule fresh weight). The thawed extract was filtered through Miracloth into a glass beaker on ice. The nodule residue was re-extracted in a mortar with a pestle in extraction buffer (2 ml buffer/g nodule fresh weight). The second nodule extract was filtered through Miracloth and combined with the first extract. The combined extracts were centrifuged at 5000 *g* for 15 min at 4° C in a Sorvall SS-34 rotor. After centrifugation the pellets were discarded, and the supernatant fractions were centrifuged at 100,000 *g* for 1 h at 4° C in a Beckman Ti 50 rotor. The supernatant fractions were discarded, and the pellets were resuspended in 20 mM MOPS-NaOH, pH 7.0, 0.5 mM EGTA (1 ml buffer/g nodule fresh weight). The resuspended membrane pellets were subjected to analysis by ELISA using the protocol described above with the following modifications.

Serial dilutions of 0.1-20 μg total membrane protein were made in 50 mM sodium carbonate, pH 9.5 and were adsorbed to a microtiter plate overnight at 4° C. ELISA incubation and washing protocols were as described above. Affinity-purified anti-CK-15 antibody (1.1 μg/ml) in PBS, 0.5% (v/v) normal goat serum (50 μl/well) was used in the primary incubation.

The analysis of the tissue distribution of nodulin 26 protein was performed by Western blot analysis. Twenty-eight day post-inoculation soybean plants were dissected into nodules, roots, stems, leaves, and apices. These tissues were frozen in liquid nitrogen and were ground in a mortar with a pestle. The samples were thawed in 20 mM MOPS-NaOH, pH 7.0, 10 mM EDTA, 10 mM $MgSO_4$, 10 mM DTT, 1% (w/v) PVP-40, 1% (w/v) BSA, 20 mM Na-isoascorbate, 1 mM PMSF, 1 μM leupeptin, and 1 μM pepstatin (3 ml buffer/g sample fresh weight). The thawed extracts were filtered through Miracloth into glass beakers on ice and were centrifuged at 5000 *g* for 10 min at 4° C in a Sorvall SS-34 rotor. The pellets were discarded, and the supernatant fractions were centrifuged at 100,000 *g* for 1 h at 4°C in a Beckman Ti 60 rotor. The supernatant fractions were discarded, and the pellets were rinsed in 10 mM MOPS-NaOH, pH 7.0 (3 ml buffer/g sample fresh weight). The pellets were resuspended in 7.5 mM sodium phosphate, pH 7.5, 1 M KI, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 μM leupeptin, 1 μM pepstatin (3 ml buffer/g sample fresh weight). The resuspended pellets were incubated for 30 min at room temperature. The samples

were centrifuged at 100,000 *g* for 1 h at 4° C in a Beckman Ti 60 rotor. The supernatant fractions were discarded, and the pellets were rinsed with 10 mM MOPS-NaOH, pH 7.0. The final pellets were resuspended in 10 mM MOPS-NaOH, pH 7.0. Equal amounts of protein from each sample (10 μg total protein) were analyzed by SDS-PAGE and Western blotting, with affinity-purified anti-CK-15 antibody.

Immunoelectron microscopy was performed by fixing and embedding 6 wk old soybean nodules as described by VandenBosch & Newcomb (1988). Briefly, nodules were fixed with 3% (v/v) glutaraldehyde in 50 mM phosphate buffer, pH 6.8. The samples were dehydrated by one wash each with 30%, 50%, 70%, 90% (v/v) ethanol in H_2O, and three washes with 100% ethanol (10 min/wash). After dehydration, the samples were embedded in LR White resin using heat polymerization. For immunogold labeling, sections on grids were blocked by incubation for 15 min in 1% (w/v) BSA, 1% (v/v) normal goat serum, 0.05% (v/v) Tween 20 and 0.02% (w/v) sodium azide, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl (blocking buffer). Sections were incubated for 1 h in anti-nodulin 26 IgG (44 μg/ml) in blocking buffer. Sections were then incubated in 15 nm goat-anti-rabbit gold diluted 1:25 or 1:50 in blocking buffer. Following immunogold labeling, sections were post-stained with 2% (w/v) aqueous uranyl acetate for 10 min and lead citrate (Reynolds, 1963) for 1 min. Sections were examined using a Zeiss 10C transmission electron microscope, operated at 80 kV.

Miscellaneous methods

Protein concentrations were determined by the method of Bradford (1976) or by the method of Smith *et al.* (1985). SDS-PAGE was performed by using the buffer system described by Laemmli (1970). Automated Edman degradation was performed on 55 pmol of HPLC-purified SM phosphopeptide by using an Applied Biosystems Model 473 automated peptide sequencer. The pth-amino acid products were analyzed by online HPLC as described by Hewick *et al.* (1981). Amino acid composition analysis was performed on acid hydrolysates as described by Bidlingmeyer *et al.* (1984). Calmodulin concentrations were determined by RIA as described by Oh & Roberts (1990). Reversible phosphorylation/dephosphorylation of nodulin 26 in intact symbiosomes and malate transport assays were performed as described in Ou Yang *et al.* (1991).

CHAPTER III

RESULTS

Detection and localization of nodulin 26 protein in soybean root nodules

In order to detect and localize the product of the nodulin 26 gene, the deduced amino acid sequence of nodulin 26 (Fortin *et al.*, 1987; Sandal & Marker, 1988) was examined and a synthetic peptide (CK-15) was produced which corresponded to the last 14 carboxyl-terminal amino acids of nodulin 26. The peptide also possessed an amino terminal cysteine to facilitate conjugation to a carrier protein for immunization (Fig. 5). Several observations suggested that this sequence was a good choice for the production of a site-specific antibody. A search of the National Biomedical Research Foundation Protein Identification Resource data base failed to identify a sequence homologous to this region suggesting that this sequence may be unique to nodulin 26. Furthermore, hydropathy analysis showed that this region is hydrophilic, and preliminary topology studies suggested that it was exposed to the cytoplasmic side of the membrane (Miao *et al.*, 1992). Overall, these observations suggested that this peptide is suitable for generating site-specific anti-nodulin antibodies. CK-15 was conjugated to ovalbumin, and the conjugate was used to raise antibodies in rabbits.

Purified SM were probed for nodulin 26 by Western blotting with anti-CK-15 antibodies. The blot revealed a single band migrating with an apparent molecular weight of

A.

```
1                                   ↓                    31
M A D Y S A G T E S Q E V V V N V T K N T S E T I Q R S D S L

32            ↓                                          62
V S L P F L Q K L V A E A V G T Y F L I F A G C A S L V V N E

63                                                       93
N Y Y N M I T F P G I A V I W G L V L T V L V Y T V G H I S G

94                                                      124
G H F N P A V T I A F A S T R R F P L I Q V P A Y V V A Q L L

125                                                     155
G S I I A S G T L R L L F M G N H D Q F S G T V P N G T N L Q

156                                                     186
A F V E F V I M T F F L M F V I C G V A T D N R A V G E L A G

187                                                     217
I A I G S T L L L N V I I G G P V T G A S M N P A R S L G P A

218                                                     248
F V H G E Y E G I W I Y L L A P V V G A I A G A W V Y N I V R

249    ↓             ↓    *     ↓          271
Y T D K P L S E I T K S A S F L K G R A A S K
```

B.

```
     ↓    *     ↓
C T K S A S F L K G R A A S K
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Figure 5. **Amino acid sequence of nodulin 26 and CK-15.** **A**, Deduced amino acid sequence of nodulin 26 (Fortin *et al.*, 1987; Sandal & Marker, 1988); **B**, amino acid sequence of CK-15. The region of homology between the COOH terminus of nodulin 26 and CK-15 is underlined. Asterisks show the sites of phosphorylation by CDPK on each molecule (Weaver & Roberts, 1992). Arrows indicated the predicted sites of cleavage for Lys-C on each molecule. Amino acids in **bold** indicate serine residues that occur within R/K-X-X-S sequences.

27,000 (Fig. 6B). This corresponded to a major protein band observed in silver-stained SDS-gels (Fig. 6A). Therefore, anti-CK-15 antibodies specifically recognized a major SM protein with a molecular weight close to that predicted from the deduced amino acid sequence of nodulin 26 (Sandal and Marker, 1988; 1990).

In order to determine the tissue specificity of nodulin 26 protein expression, anti-CK-15 antibodies were used to probe samples of 100,000 *g* membrane pellets from a variety of soybean tissues including roots, leaves, stems, apices, and nodules. As shown in Figure 7, only the membrane fraction from nodules exhibited any detectable immunoreactivity with anti-CK-15 antibodies. Furthermore, consistent with the proposed membrane location of nodulin 26, immunoreactivity was not detected in any of the soluble fractions tested. These data support the results of the cDNA screening which suggested that nodulin 26 is a nodule-specific protein, as well as Western blots with isolated SM (Weaver *et al.* 1991) and *in vitro* translation results that suggested that it is found in the SM (Fortin *et al.*, 1987).

Although the results of the Western blots showed that nodulin 26 was found specifically in nodules, and that it is a major component of the SM, it was not clear whether nodulin 26 was localized exclusively to the SM. In order to determine this, antibodies raised against gel-purified nodulin 26 were used to probe fixed and embedded sections of nitrogen-fixing

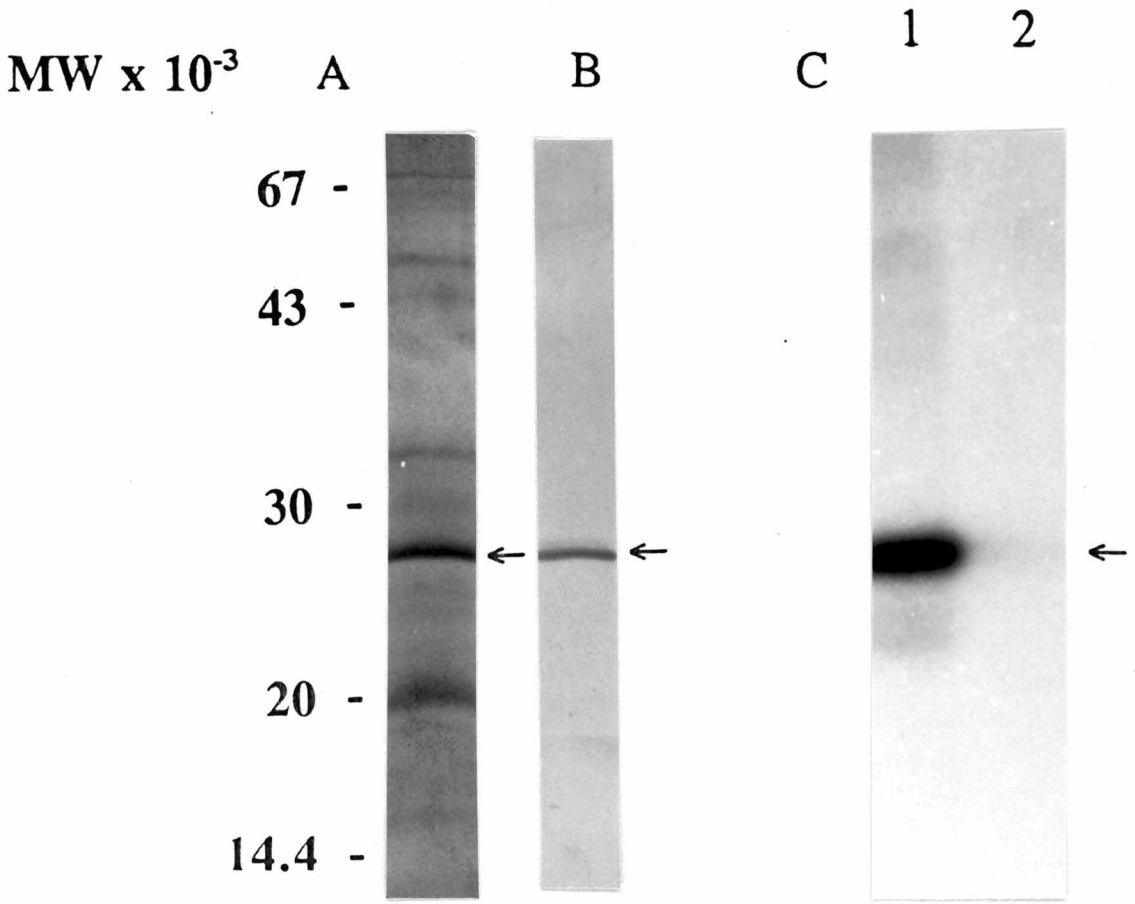


Figure 6. Analysis of SM fractions for anti-CK-15 immunoreactivity and calcium-dependent phosphorylation. Purified SM were analyzed for reactivity with anti-CK-15 antibodies and for calcium-dependent phosphorylation. The membranes were subjected to SDS-PAGE on 14% (w/v) polyacrylamide gels. **A.** silver-stained gel of SM proteins (4 μg total protein). **B.** Western blot of SM protein probed with anti-CK-15 antibody (24 μg total protein). **C.** autoradiogram of SM proteins incubated with γ-^{32}P-ATP in the presence of 0.5 mM $CaCl_2$ (lane **1**) or 1 mM EGTA (lane **2**). Arrows show a major SM protein corresponding to the position of the immunoreactive and radiolabeled band.

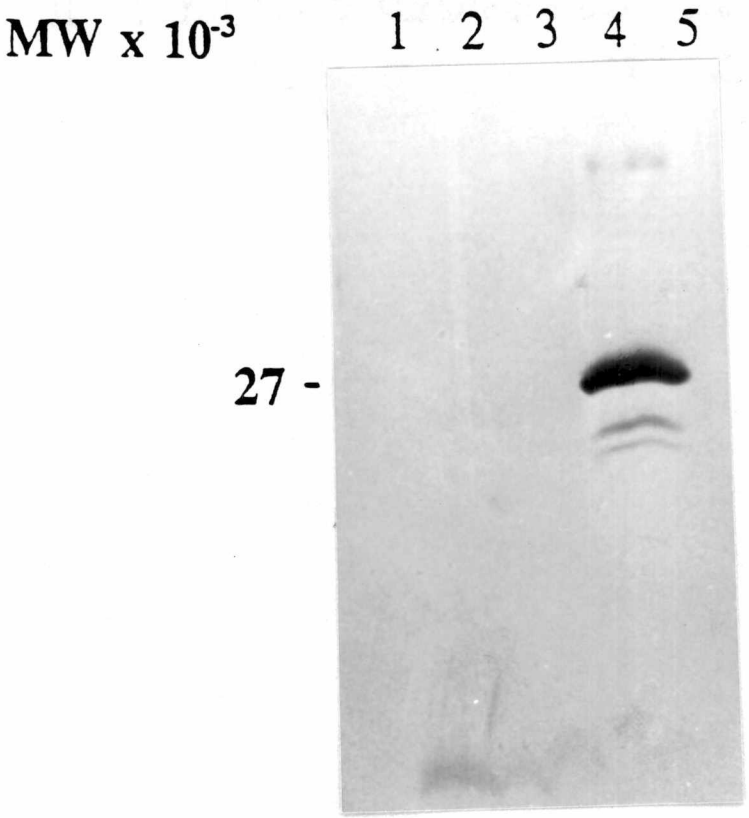


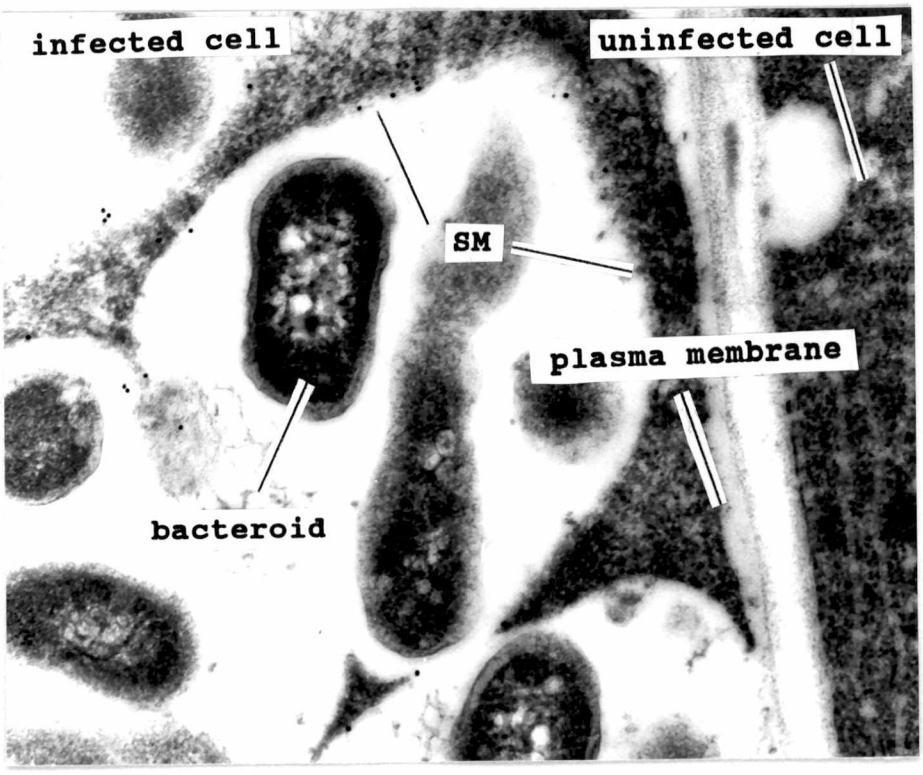
Figure 7. Distribution of anti-CK-15 immunoreactivity in soybean tissues. 100,000 *g* membrane pellets were separated by SDS-PAGE on 15% (w/v) polyacrylamide gels and were subjected to Western blot analysis with anti-CK-15 antibodies. Lane **1**, roots; lane **2**, leaves; lane **3**, apices; lane **4**, nodules; lane **5**, stems. 10 μg total protein/lane. The two lower molecular weight bands are probably proteolytic degradation products of nodulin 26 since they increase in intensity with prolonged incubation of nodulin in the absence of protease inhibitors.

soybean root nodules. Analysis of the resulting immunoelectron micrographs (Fig. 8) demonstrated that anti-nodulin 26 immunoreactivity was observed in infected but not in uninfected soybean root nodule cells. Furthermore, this immunoreactivity was specifically localized to the SM, and was not associated with other membranes of the infected nodule cells. These data constitute the first direct demonstration that nodulin 26 is found only in the SM.

As discussed previously, nodulin genes show temporal differences in their expression. Some of these genes are expressed at the onset of symbiosis (ENODs), while others are expressed after nodules have been formed or at the time that nitrogen fixation begins (NODs)(reviewed in Franssen *et al.*, 1992). Furthermore, the expression of some nodulins is blocked when plants are infected with mutant rhizobia that are unable to form effective, nitrogen-fixing nodules (Morrison & Verma, 1987). These mutants form nodules that contain cells with large numbers of membrane-bound vesicles that do not contain bacteroids (Fig. 9). The phenotype of these nodules (bar$^-$) results from a defect in the release of the rhizobia from the infection thread (Roth & Stacey, 1989). The expression of nodulin 26 protein in these nodules was investigated by preparing 100,000 *g* membrane fractions from soybean nodules arising from infection by wild-type and three lines of bar$^-$ mutant rhizobia (ML126, ML146, and ML150)(So *et al.*, 1987). The membranes were analyzed for the expression

Figure 8. Immunoelectron micrographs of soybean root nodule sections. 42 day old nodules were fixed, embedded, and stained with immunogold particles as described in Materials and Methods. **A**, nodule section incubated with anti-nodulin 26 antibodies (magnification 16,000 x). **B**, nodule section incubated with pre-immune serum (magnification 16,000 x). Immunoelectron micrographs provided by Dr. Kate VandenBosch, Texas A & M University.

A


infected cell
uninfected cell
SM
plasma membrane
bacteroid

B

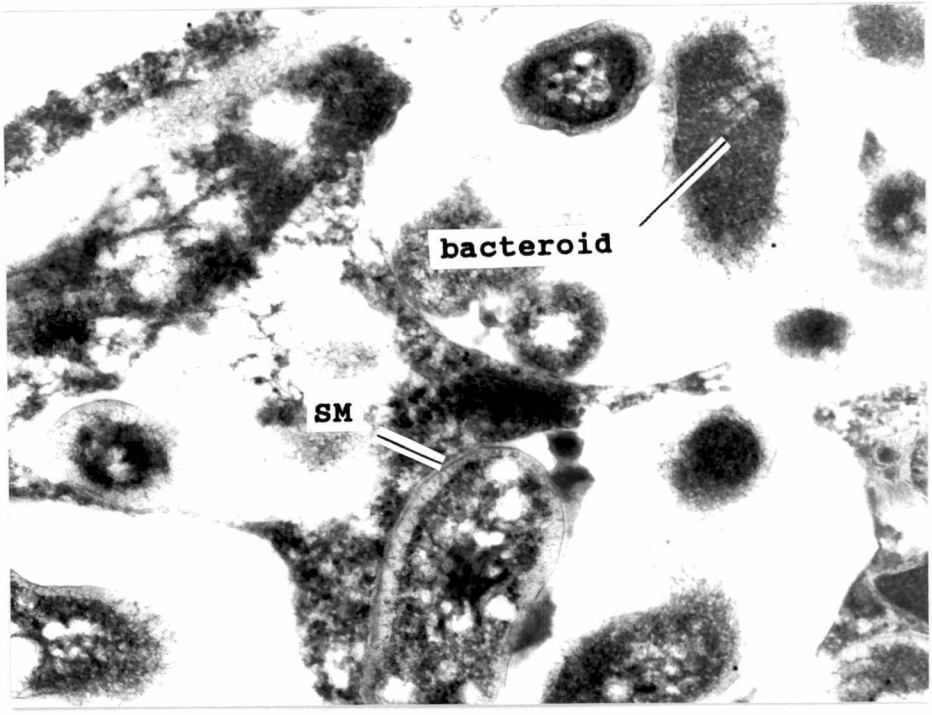
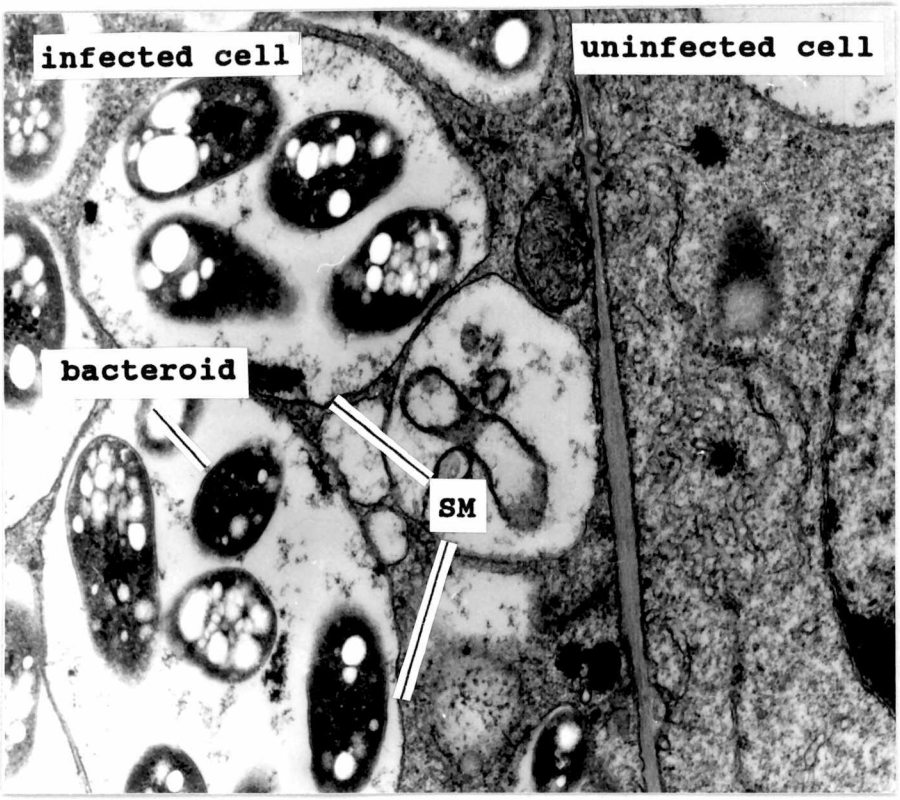

bacteroid
SM

Figure 9. Electron micrographs of wild-type and mutant soybean root nodules. Sections are from 28 day post-inoculation soybean nodules. **A**, wild-type *Bradyrhizobium japonicum* strain 61A101C (magnification 20,000 x). **B**, mutant *Bradyrhizobium japonicum* strain ML126 (magnification 8,400 x). Electron micrographs provided by Dr. Evans Roth, Department of Zoology, University of Tennessee.

A



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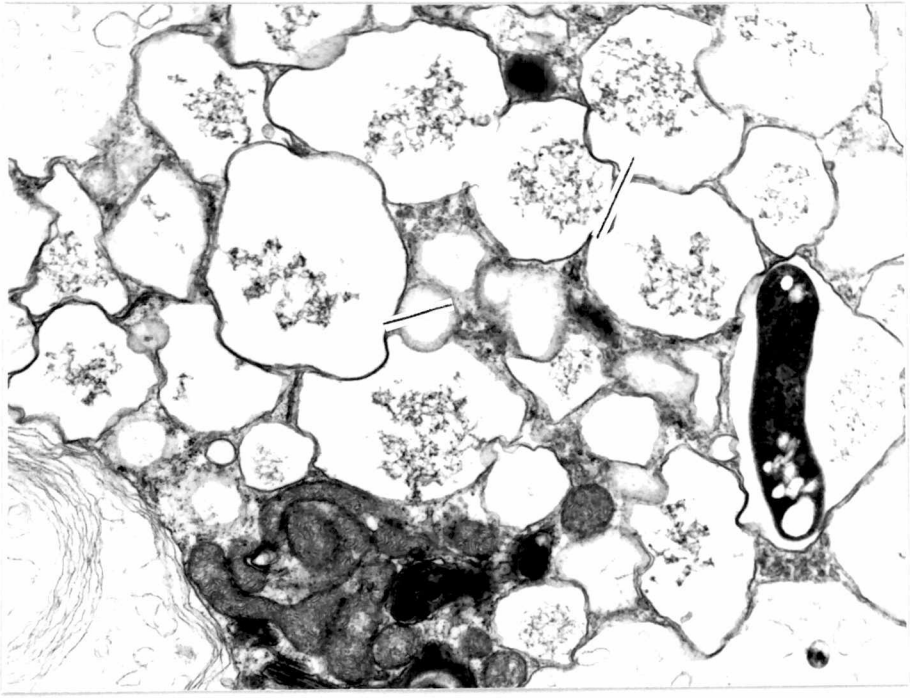


of nodulin 26 protein by ELISA with anti-CK-15 antibodies. These analyses revealed that nodulin 26 protein was expressed at comparable levels in all samples tested (Fig. 10). This observation demonstrates that nodulin 26 expression is not dependent on the successful establishment of a nitrogen-fixing symbiosis, and that its expression is signaled by an event prior to the release of the rhizobia into the symbiosome or the onset of nitrogen fixation. Thus, the division of nodulins into two broad classes of "early" and "late" nodulins may be an oversimplification.

Detection and purification of a calcium-dependent protein kinase from soybean root nodules

Examination of the deduced amino acid sequence of nodulin 26 revealed five sequences (Fig. 5) that resemble sites phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II from animals (Kemp & Pearson, 1990) and CDPKs from higher plants and algae (reviewed in Roberts & Harmon, 1992). Two of these sites are found in CK-15. To determine whether or not CK-15 is phosphorylated by a protein kinase in soybean root nodules, CK-15 was incubated in a standard protein kinase reaction mixture containing soybean root nodule extracts. Ca^{2+}-dependent CK-15 kinase activity was detected (Table I). This activity was purified 1570-fold from extracts of soybean root nodules (Table II) and it possessed a terminal specific activity of 1400 nmol phosphate incorporated/min/mg protein (Table II). This value was similar to the specific activity

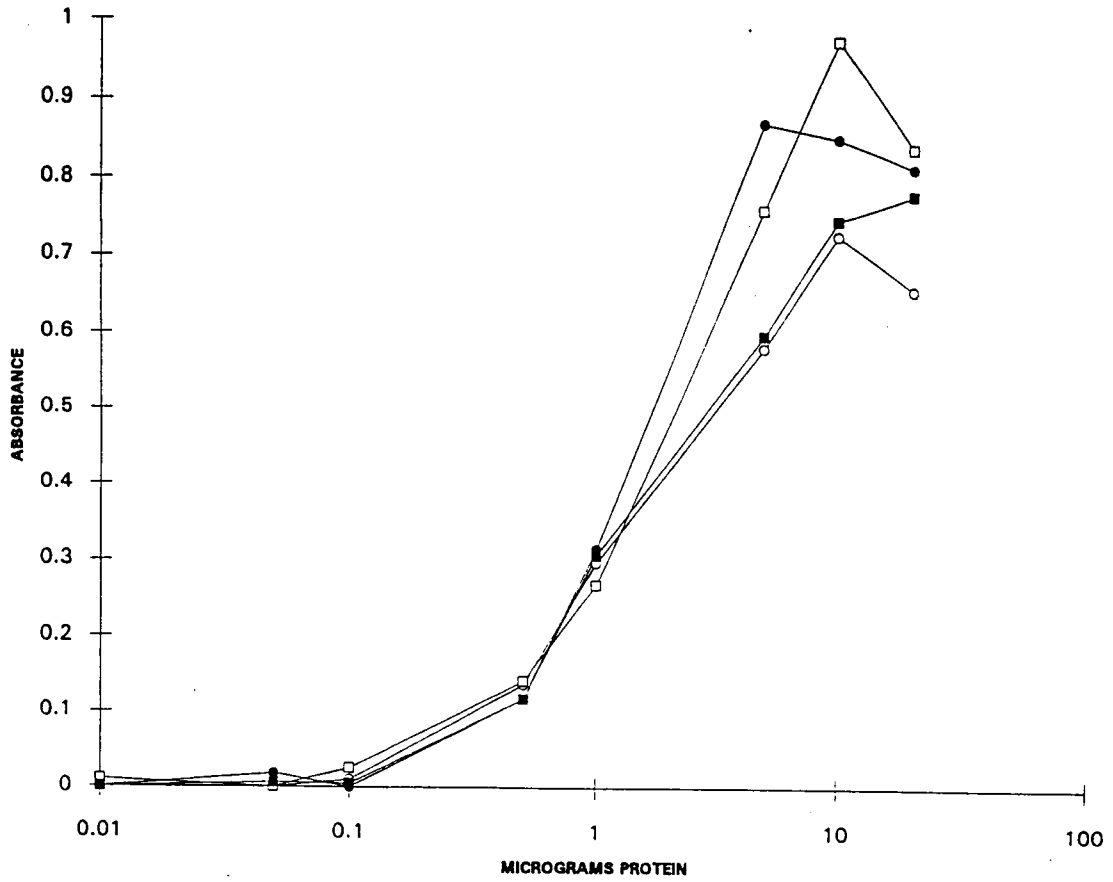


Figure 10. Comparison of nodulin 26 protein levels in membrane fractions from wild-type and mutant soybean nodules. 100,000 *g* membrane pellets were prepared as described in Materials and Methods. ELISAs were done on various amounts of membrane proteins from of wild-type and mutant soybean nodules using anti-CK-15 antibodies. ○, wild-type (61A101C); ●, ML126; □, ML146; ■, ML150. The absorbance at 540 nm is plotted against the amount of membrane protein.

Table I. Detection of CK-15 kinase activity in soybean root nodules.

Substrate	kinase activity[a]	
	Ca	EGTA
	nmol ^{32}P incorporated/min/mg protein	
none	0.073	0.051
130 μM CK-15	0.894	0.164

[a]Activity shown was obtained using standard assay procedures in the presence of: Ca, 1 mM $CaCl_2$; EGTA, 1 mM EGTA.

Table II. Summary of purification of CK-15 kinase from soybean root nodules[a]

Step	Total Activity[b]	Yield	Specific Activity[c]	Purification
		%		-fold
Extract	687	100	0.89	1
100,000 *g* supernatant	524	82	1.28	1.4
8-28% PEG	298	47	2.49	2.8
DEAE cellulose	105	17	9.39	10.5
Sephacryl 300 HR	73.3	12	14.4	16.1
Mono Q	44.0	7.2	1400	1570

[a]The results shown are from the preparation of enzyme from 40 g of root nodules. Assays were performed in the presence of 129 μM CK-15 and saturating Ca^{2+} (0.3-0.5 mM) under standard protein kinase assay conditions.

[b]Expressed as nmol ^{32}P incorporated/min.

[c]Expressed as nmol ^{32}P incorporated/min/mg protein.

reported for the CDPK purified from soybean tissue culture cells (1700 nmol phosphate incorporated/min/mg protein) (Putnam-Evans *et al.*, 1990). The purity of the preparation was examined on silver-stained SDS-gels. These gels revealed one major band migrating with an apparent molecular weight of 60,000 and two clearly resolved, higher molecular weight, minor bands (Fig. 11A). To determine if the major band (60,000 MW) was the protein kinase, the purified preparation was subjected to SDS-PAGE in gels containing histone IIIs. This is a lysine-rich histone preparation that is commonly used as a substrate for CDPKs (Roberts & Harmon, 1992). Protein kinase activity was detected by blotting to nitrocellulose, *in situ* renaturation of the kinase, incubation in the presence of γ-^{32}P-ATP, and visualization of radioactive products by autoradiography. This analysis revealed a single radioactive band migrating with an apparent molecular weight of 60,000 (Fig. 11B). Furthermore, monoclonal antibodies raised against purified soybean tissue culture cell CDPK (Putnam-Evans *et al.*, 1990) also recognize the 60,000 MW protein (Fig. 11C). These observations suggest that the CK-15 kinase is the major 60,000 MW protein in the preparation and that the soybean root nodule enzyme is related to the CDPK family of Ca^{2+}-dependent protein kinases.

The properties of the CK-15 kinase were evaluated based on its solubility, sensitivity to ionic strength, Ca^{2+}-dependence, and sensitivity to exogenously applied effectors

of other Ca^{2+}-dependent protein kinases. The purified activity appeared to be soluble based on the fact that it did not sediment upon centrifugation at 100,000 *g* for 1 h (Table II). After centrifugation, the activity appeared to be completely dependent on the presence of CK-15. The ability of the kinase to phosphorylate CK-15 was substantial even in the presence of 0.15 M KCl indicating that CK-15 is a substrate under conditions of physiological ionic strength (Fig. 12), thus supporting the relevance of CK-15 as a substrate for the study of this kinase activity.

The Ca^{2+}-dependence of activation was measured by conducting assays in the presence of varying concentrations of free Ca^{2+} by using the Ca^{2+}/EGTA buffer system described in Materials and Methods. The CK-15 kinase exhibited a $K_{0.5}$ for Ca^{2+} of 0.4 μM (Table III and Fig. 13). This Ca^{2+} concentration is within the known range of physiological fluxes in cytosolic Ca^{2+} measured in plants (Williamson & Ashley, 1982). The Ca^{2+} stimulation of the kinase varied with each preparation and ranged from 10 to 30-fold. Despite this variation, the results suggest that the CK-15 kinase is substantially stimulated by physiologically relevant concentrations of Ca^{2+}.

The effect of exogenously applied calmodulin, at concentrations up to 100 nM, was determined by the addition of spinach calmodulin to the CK-15 kinase assay mixture. This concentration of calmodulin should be adequate to stimulate

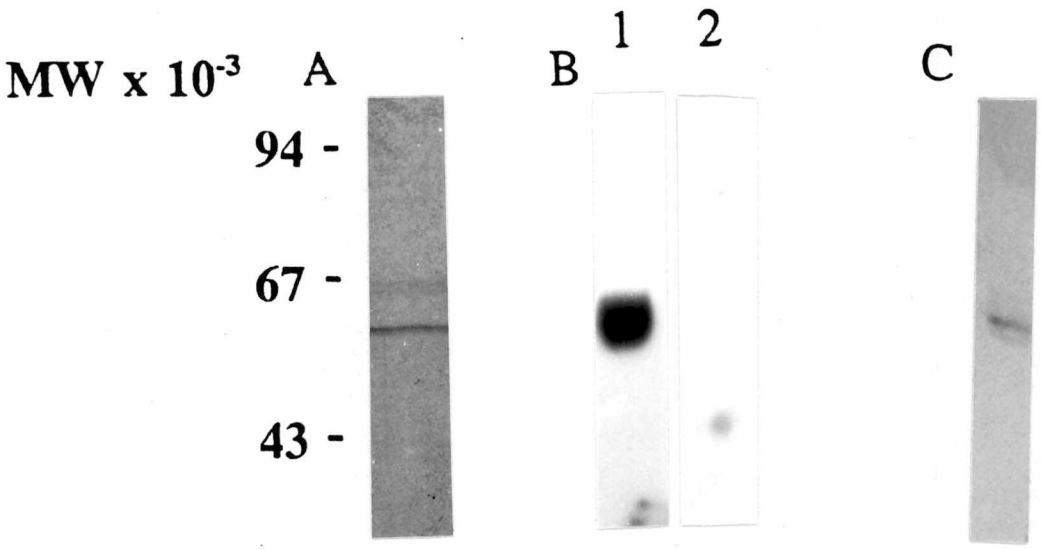


Figure 11. SDS-PAGE analysis of the CK-15 kinase from soybean root nodules. The enzyme was purified as described in the Materials and Methods. **A**, silver-stained gel of purified CK-15 kinase from nodules. **B**, autoradiogram of a protein kinase activity stain of CK-15 kinase with histone IIIs as substrate. **1**, activity stain conducted in the presence of 0.5 mM $CaCl_2$; **2**, activity stain conducted in the presence of 1 mM EGTA. **C**, Western blot of CK-15 kinase probed with anti-soybean tissue culture cell CDPK monoclonal antibodies.

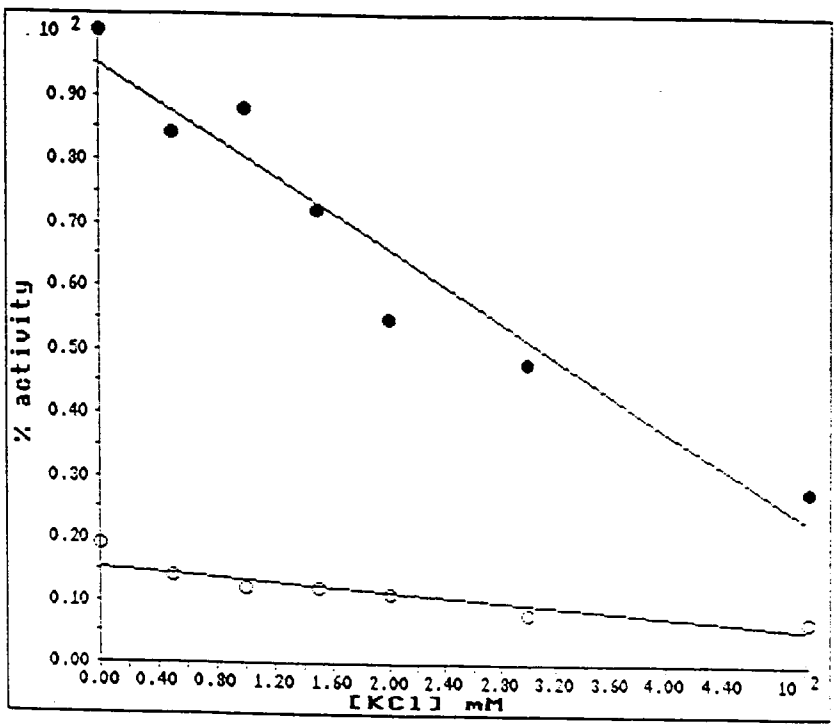


Figure 12. Effect of ionic strength on the soluble CK-15 kinase purified from soybean root nodules. Assays were performed with 129 μM CK-15 at pH 7.0 and: 0, 50, 150, 200, 300, and 500 mM KCl in the presence of 0.5 mM $CaCl_2$ (•), or 1 mM EGTA (○).

Table III. Dependence of purified CK-15 kinase on calcium and calcium effector molecules

Conditions[a]	Protein Kinase Activity[b]	
	+CK-15	-CK-15
	μmol ^{32}P incorporated/min/mg protein	
Ca^{2+}	1.28 ($K_{0.5}$=0.4 μM)[c]	0
EGTA	0.16	n.d.
Ca^{2+}/calmodulin	1.48	n.d.
Ca^{2+}/PS and DAG	1.48 ($K_{0.5}$=0.5 μM)	n.d.

[a]The conditions are: Ca^{2+}, 0.5 mM $CaCl_2$; EGTA, 0.5 mM EGTA; Ca^{2+}/calmodulin, 0.5 mM $CaCl_2$ and 100 nM spinach calmodulin; Ca^{2+}/PS and DAG, 0.5 mM $CaCl_2$, 24 μM phosphatidylserine, and 0.64 μM 1,2 dioleylglycerol.

[b]+CK-15, activity with 129 μM CK-15; -CK-15, activity with no added substrate; values represent the average of two determinations. n.d., not determined.

[c]$K_{0.5}$ values represent the calcium concentration required for half-maximal stimulation of the enzyme.

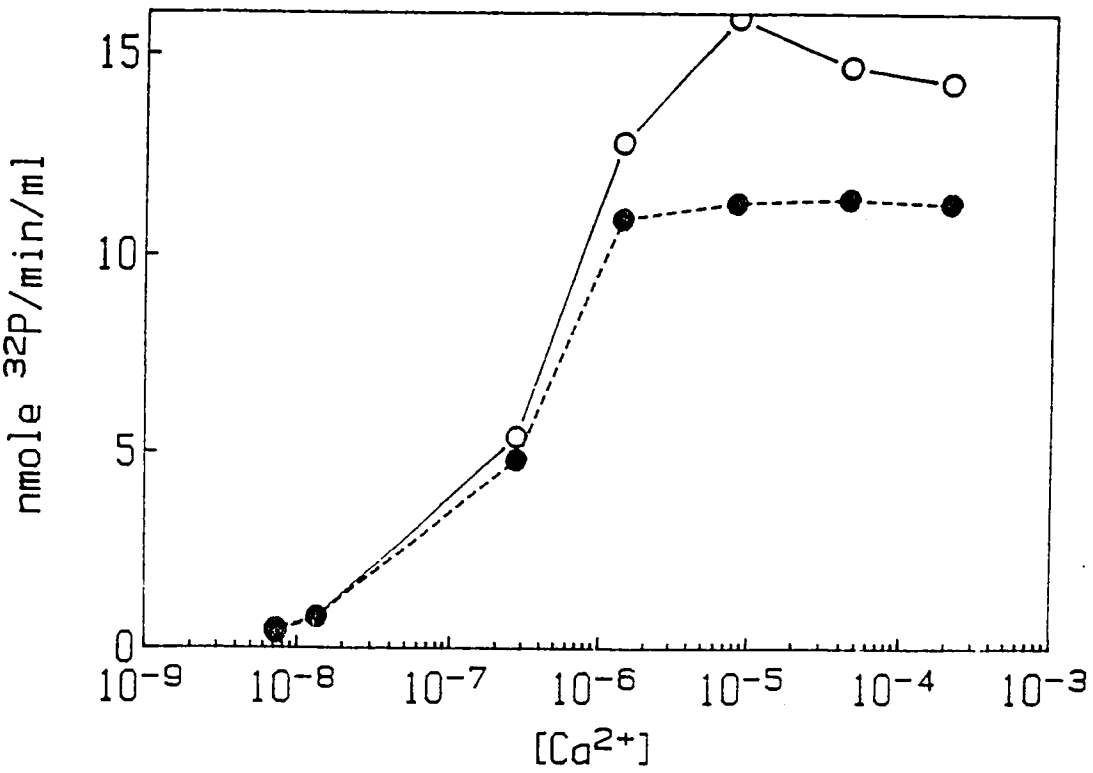


Figure 13. Calcium dependence of purified CK-15 kinase from soybean root nodules. Assays were performed under standard conditions in the presence of various concentrations of Ca^{2+} and the presence (O) or absence (•) of 24 μM phosphatidylserine and 0.64 μM 1,2 dioleoylglycerol.

most calmodulin-regulated enzymes (K_d for calmodulin usually ~1 nM)(Cohen & Klee, 1988; O'Neil & Degrado, 1990). The results show that the addition of calmodulin did not significantly affect the kinase activity (Table III). Further, the lack of calmodulin stimulation could not be attributed to the presence of endogenous calmodulin. The profiles of kinase and calmodulin elution from the Mono Q column showed that the kinase eluted as a narrow peak from the Mono Q column that was well resolved from the calmodulin peak (Fig. 14). This kinase preparation was demonstrated to be free of calmodulin by RIA analysis.

The effects of known lipids effectors on the activity of the kinase were also determined. The addition of phosphatidylserine and diacylgycerol (activators of the Ca^{2+}-dependent protein kinase C, Kikkawa *et al.*, 1982) had little effect on the maximum activity of the kinase and did not affect the $K_{0.5}$ for Ca^{2+} stimulation (Table III, Fig. 13).

These data demonstrate that the soluble CK-15 kinase from soybean root nodules is not dependent on calmodulin nor lipids for maximum activity. The micromolar sensitivity to Ca^{2+} and the lack of a requirement of the enzyme for calmodulin and lipid effectors are consistent with the structure of cloned CDPKs which predicts four EF-hand Ca^{2+}-binding sites (Harper *et al.*, 1991). These results, combined with the immunochemical evidence presented above, indicate that the CK-15 kinase is a member of the CDPK family.

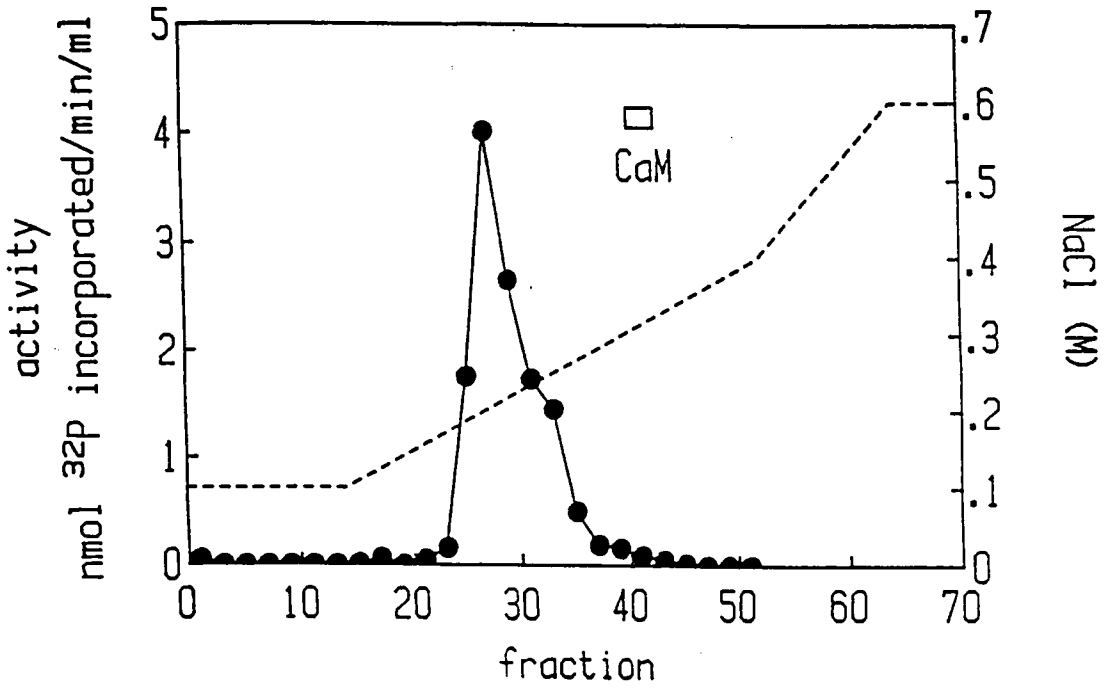


Figure 14. Mono Q chromatography of CK-15 kinase. CK-15 kinase (0.49 mg of enzyme purified through the Sephacryl 300 HR step, Table II) was chromatographed on a Mono Q column attached to a FPLC. Active fractions were identified by the standard protein kinase assay procedure. The presence of calmodulin was determined by RIA as described by Oh and Roberts (1990). Closed circles indicate points of kinase elution; the open box indicates the position of calmodulin elution; the dashed line indicates the NaCl gradient (0-0.7 M).

Detection and partial purification of a SM-associated calcium-dependent protein kinase

Previous studies with CDPK enzymes suggest that they are often associated with membrane fractions (Klucis & Polya, 1988; Schaller & Sussman, 1988; Weaver *et al.*, 1991; Schaller *et al.*, 1992) To determine if CK-15 kinase activity was limited solely to the soluble fraction of nodule extracts, the distribution of CK-15 kinase activity was investigated in both the soluble (100,000 *g* supernatant fraction) and particulate membrane (100,000 *g* pellet) fractions of nodules and other soybean tissues. CK-15 kinase activity was detected in both the particulate and soluble fractions of all tissues tested including nodules, roots, stems, and leaves (Table IV). Furthermore, CK-15 kinase activity was also associated with purified SM at a specific activity that is comparable to other membrane fractions (Table IV). These findings indicate that, unlike nodulin 26, the CK-15 kinase activity is widespread in the plant. This observation is consistent with previous studies of the localization of CDPKs (reviewed in Roberts & Harmon, 1992) and it suggests a broad role for this kinase in mediating the effects of Ca^{2+} signals. These data also identify a CK-15 phosphorylating activity that is associated with the SM, which is also the location of nodulin 26. Thus, nodulin 26 may be the target of this SM-associated protein kinase.

To test this hypothesis, a more thorough investigation of

Table IV. Distribution of calcium-dependent CK-15 kinase in subcellular fractions of soybean plants

Tissue	CK-15 Protein Kinase Activity[a]		
	S100[b]	P100	SM
	nmol ^{32}P incorporated/min/mg protein		
Nodule	1.0	1.3	1.2
Root	1.5	1.5	-
Stem	2.9	1.9	-
Leaf	0.3	0.3	-

[a]Activity shown was obtained with 129 μM CK-15 and 0.5 mM $CaCl_2$ under the standard assay conditions. The value was corrected for non-CK-15 protein kinase activity by subtracting the value obtained with $CaCl_2$ in the absence of CK-15. The values are the average of two different determinations.

[b]S100, soluble fractions obtained after centrifugation of extracts at 100,000 *g* (see Materials and Methods); P100, particulate membrane fraction that sediments between 6,000 to 100,000 *g*; SM, purified SM prepared from isolated symbiosomes.

the SM-associated CK-15 kinase was conducted. To determine if the kinase was peripherally associated with the SM, purified SM were washed with a buffer containing 1 M KI to remove extrinsic membrane proteins. CK-15 kinase assays of the KI-stripped membranes revealed that the kinase activity was resistant to removal by this agent (Table V). After treatments with other agents (100 mM NaOH or 8 M urea) used to remove extrinsic membrane proteins, CK-15 kinase still remained associated with the SM. However, CK-15 kinase activity could be extracted from SM by the zwitterionic detergent CHAPS (Table V). Therefore, the SM-associated CK-15 kinase activity appeared to be integrally associated with the SM; perhaps via a hydrophobic amino acid domain or by means of some other posttranslationally attached hydrophobic anchor.

The ability of the SM-associated CK-15 kinase activity to be solubilized allowed for partial purification and characterization of the activity. The CHAPS-solubilized kinase was subjected to Mono Q ion-exchange chromatography. This resulted in a 9.3-fold purification after solubilization (Table VI). The Ca^{2+}-stimulation of the Mono Q-purified SM-associated kinase was 400-fold (Table VI).

The soluble and SM-associated CK-15 kinases were compared by SDS-PAGE and Western blotting with monoclonal antibodies raised against the soluble CDPK purified from soybean tissue culture cells (Putnam-Evans *et al.*, 1990). When equal CK-15 kinase activities were analyzed by this means, the anti-CDPK

Table V. Characterization of the properties of the membrane association of the SM-associated CK-15 kinase

Treatment	Activity[a]	% recovery
Untreated SM	0.29	100
KI washed SM	0.21	72
CHAPS-solubilized SM	0.23	79

[a]Activity (expressed as pmol ^{32}P incorporated/min/μl) was obtained in the presence of 129 μM CK-15 and 0.5 mM $CaCl_2$ under standard assay conditions.

Table VI. Summary of the partial purification of the SM-associated CK-15 kinase

Step	Specific Activity[a]	Yield %	Purification -fold	Ca^{2+}/E^{b}
CHAPS-solubilized	1.3	100	1	∞
SM Mono Q	12	147	9.3	400

[a]Activity expressed as nmol ^{32}P incorporated/min/mg protein in the presence of 129 μM CK-15 under standard assay conditions.

[b]Ca^{2+}/E shows the ratio of CK-15 kinase activity in the presence of 0.5 mM $CaCl_2$ to that obtained in 1 mM EGTA.

antibodies failed to recognize the Mono Q-purified, SM-associated kinase, whereas the soluble kinase was readily detected (Fig. 11). Based on these results, it appears that the SM-associated kinase is immunologically distinct from the soluble CK-15 kinase from soybean root nodules and the soluble CPDK from soybean tissue culture cells. The lack of cross-reactivity of the SM-associated kinase may indicate that the soluble and SM-associated kinase activities are actually different proteins.

The possibility that the SM-associated CK-15 kinase is a CDPK is supported by several observations. First, the enzyme phosphorylates CK-15 in a manner similar to the soluble enzyme. Second, SM-associated CK-15 kinase activity did not require the addition of calmodulin for Ca^{2+}-stimulation even after treatment of the SM with 1 M KI, 8 M urea, or 100 mM NaOH. These treatments would presumably remove calmodulin from the membrane fraction. Despite these studies, the unequivocal identification of the SM-associated CK-15 kinase as a CDPK remains to be done. The fact that this CDPK does not cross react with the anti-CDPK monoclonal antibodies strongly suggests that the SM-associated CK-15 kinase is structurally distinct from known soybean CDPKs. However, this is not an adequate proof that this enzyme is not a CDPK. For example, the SM-associated enzyme may not share the epitopes recognized by these monoclonal antibodies (which react with the catalytic domain of soluble soybean CDPK, Alice Harmon,

personal communication). The ultimate proof will be provided by the cloning of the cDNA for the SM-associated enzyme and the demonstration that this enzyme possesses the structural hallmarks that distinguish this class of protein kinases (Roberts, 1993). Also it will be important to determine whether, similar to the plasma membrane-associated CDPKs (Schaller & Sussman, 1988; Schaller *et al.*, 1992; Harper *et al.*, 1993), lipids modulate the SM-associated CK-15 kinase activity. Despite the uncertainty regarding the identity of the SM-associated CK-15 kinase, it possesses functional properties that are consistent with known CDPKs. Therefore, for convenience, the SM-associated CK-15 kinase will be referred to as the SM-associated CDPK.

Phosphorylation of nodulin 26 by the SM-associated CDPK

The demonstration that CK-15 was phosphorylated by a CDPK from soybean root nodules, including a SM-associated CDPK, suggested that nodulin 26 might be a CDPK substrate. To determine whether nodulin 26 was phosphorylated *in situ*, purified SM were incubated with γ-^{32}P-ATP in the presence of Ca^{2+} or EGTA under standard protein kinase assay conditions. SDS-PAGE and autoradiography of the products of the incubation revealed the Ca^{2+}-dependent phosphorylation of one major band migrating with an apparent molecular weight of 27,000 (Fig. 6C). Western blot analysis with the anti-CK-15 antibody showed a cross-reactive band with mobility identical to the phosphorylated band (Fig. 6B). These data strongly suggested

that nodulin 26 was phosphorylated by the SM-associated CDPK *in situ*.

To determine if nodulin 26 was phosphorylated *in vivo*, intact root nodules were metabolically-labeled with ^{32}P-phosphate and SM were purified and analyzed by SDS-PAGE, Western blotting, and autoradiography. Autoradiography revealed a single phosphorylated band migrating with an apparent molecular weight of 27,000 (Fig. 15). This band was recognized by anti-CK-15 antibodies (Fig. 15). Taken together, these results suggest that nodulin 26 is phosphorylated in SM *in situ*, as well *in vivo*. Thus, nodulin 26 appears to be a target of Ca^{2+}-mediated phosphorylation by CDPK in soybean root nodules.

The phosphorylation site of an endogenous substrate of a CDPK had not been previously determined. Therefore, it was of interest to elucidate which sites were phosphorylated on nodulin 26 *in vitro* and *in vivo*. Nodulin 26 contains thirty-seven serine and threonine residues including five potential sites of phosphorylation within the R/K-X-X-S sequence (Fig. 5). Two of these sites are in CK-15 (Fig. 5). Since nodulin 26 was the only detectable phosphoprotein in the SM (Fig. 6C), purified SM were used as a source of nodulin 26 for phosphorylation site analysis. To determine whether serine and/or threonine were phosphorylated, phosphorylated nodulin 26 was subjected to acid hydrolysis followed by standard phosphoamino acid analysis. The results of this determination

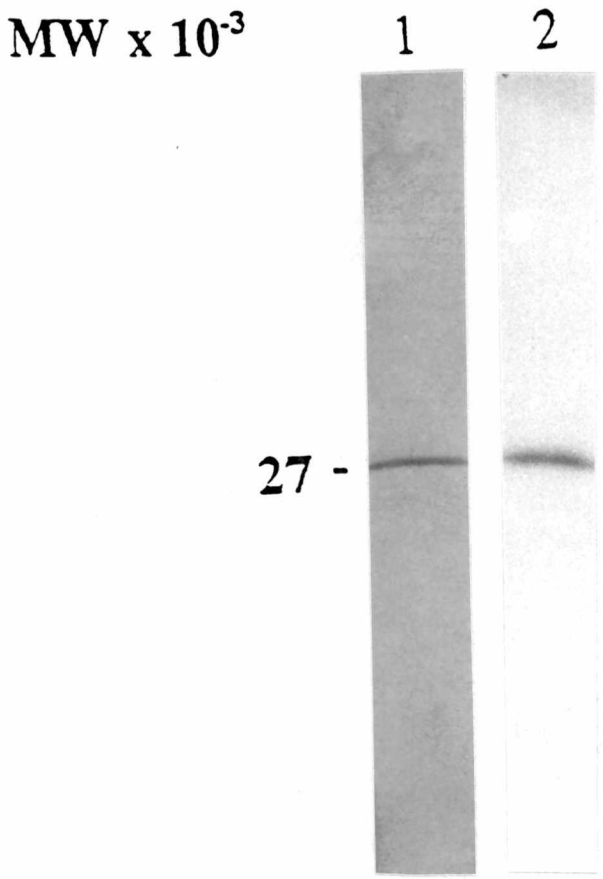


Figure 15. Analysis of SM from ^{32}P-labeled nodules for anti-CK-15 immunoreactivity. Nodules were labeled with ^{32}P-phosphate and SM were isolated and subjected to SDS-PAGE on 14% (w/v) polyacrylamide gels. **1**, Western blot analysis of SM with anti-CK-15 antibody; **2**, autoradiogram of the blot in 1 showing ^{32}P incorporation into SM proteins.

clearly indicated the presence of phosphoserine whereas phosphothreonine was not detected (Fig. 16).

For the determination of the exact serine(s) phosphorylated in nodulin 26, phosphopeptide mapping and sequence analysis were performed. Examination of the deduced amino acid sequence of nodulin 26 shows a total of 5 lysine residues, two in the hydrophilic amino-terminal portion of the molecule and three in the hydrophilic carboxyl-terminal portion of the molecule (Fig. 5). The locations of these lysines are such that digestion of nodulin 26 with the lysine-specific protease Lys-C should generate five hydrophilic peptides which contain all of the serine residues found in the R/K-X-X-S motifs (Fig. 5).

Following Lys-C digestion of SDS-solubilized, phosphorylated nodulin 26, the peptides were subjected to one and two-dimensional phosphopeptide mapping. Analysis of one dimensional TLE and TLC phosphopeptide maps revealed that single phosphopeptide spots with identical mobility were obtained from Lys-C digestions of nodulin 26 and CK-15 phosphorylated *in vitro* by the SM-associated CDPK (Fig. 17). Analysis of one dimensional TLE phosphopeptide maps of nodulin 26 metabolically labeled in intact nodules showed identical results (Fig. 18). Furthermore, the nodulin 26 and CK-15 phosphopeptides migrated identically when mixed and analyzed by two-dimensional phosphopeptide mapping (Figs. 19 and 20). These results suggest that nodulin 26 and CK-15 may have the

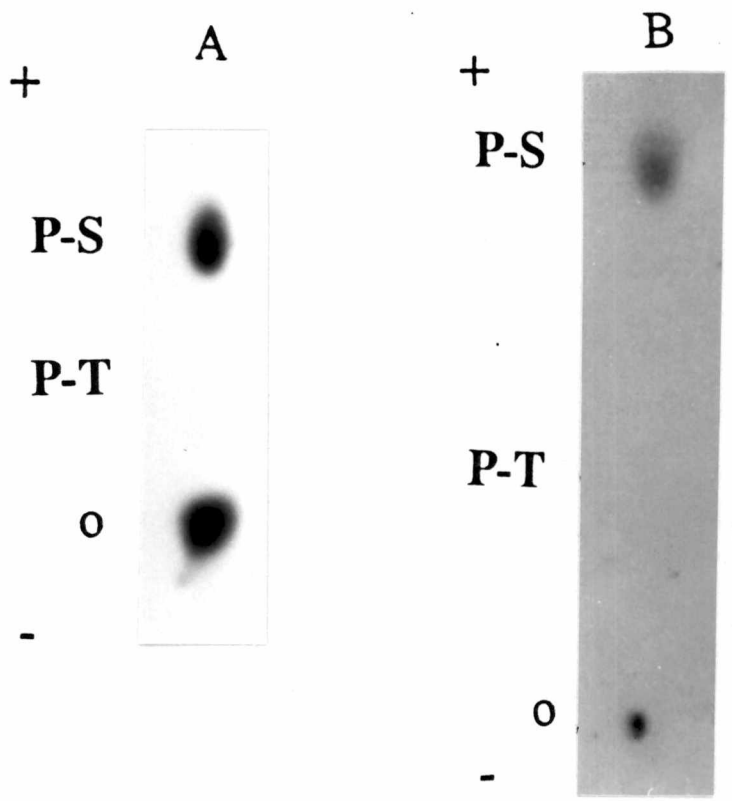


Figure 16. Phosphoamino acid analysis of phosphorylated nodulin 26. Phosphorylated nodulin 26 was subjected to acid hydrolysis *in vacuo* and was analyzed by TLE as described in Materials and Methods. **A**, nodulin 26 phosphorylated *in vitro* by the SM-associated CDPK. **B**, nodulin 26 phosphorylated in intact nodules metabolically labeled with ^{32}P-phosphate. P-T and P-S indicate the position of migration of phosphothreonine and phosphoserine standards respectively. **O**, indicates the origin of sample application.

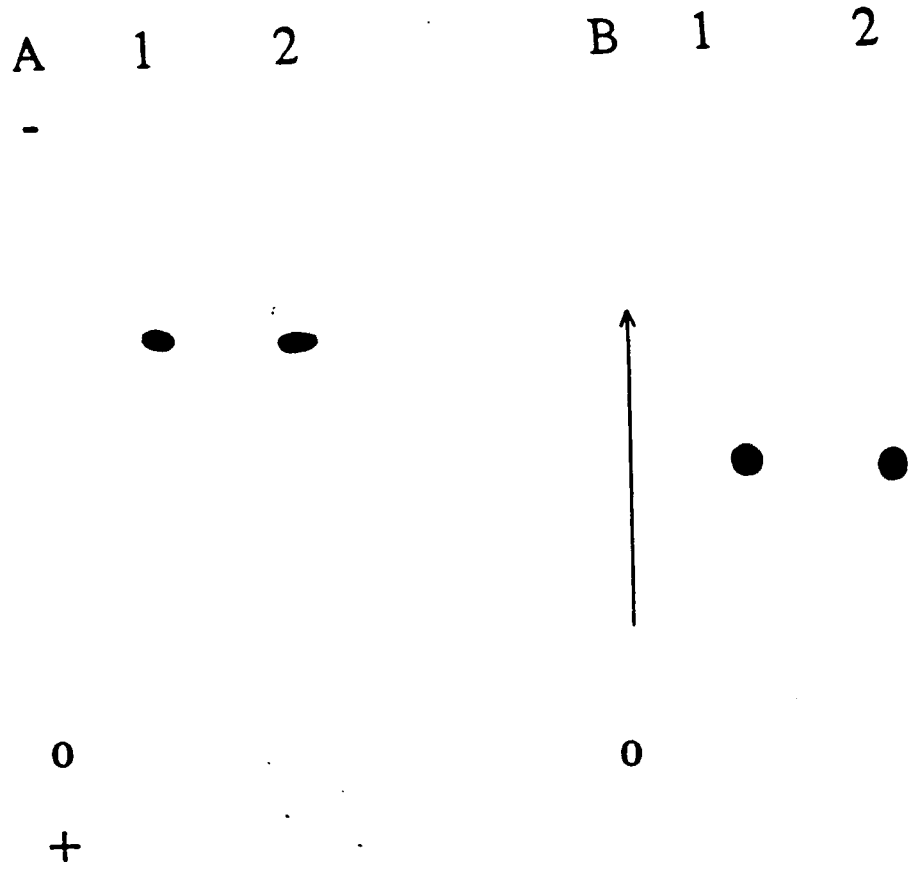


Figure 17. Phosphopeptide mapping of *in vitro*-labeled nodulin 26 and CK-15. Nodulin 26 and CK-15 were labeled *in vitro* by SM-associated CDPK, digested with Lys-C, and analyzed by TLE and TLC as described in Materials and Methods. **A**, 3000 cpm of (**1**) nodulin 26 and (**2**) CK-15 phosphopeptides were subjected to TLE and autoradiography. **B**, 3000 cpm of (**1**) nodulin 26 and (**2**) CK-15 phosphopeptides were subjected to TLC and autoradiography. **O**, indicates the origin. The arrow indicates the direction of chromatography.

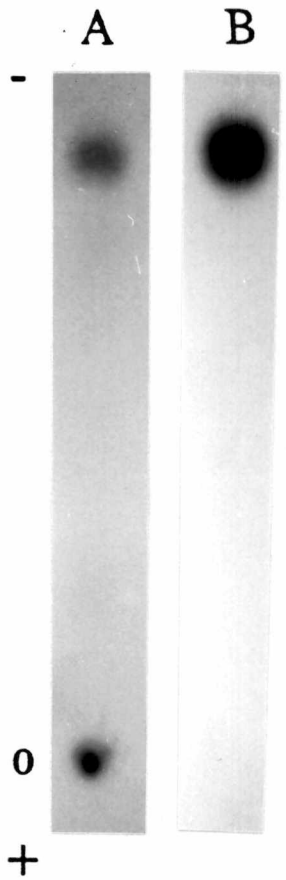


Figure 18. Phosphopeptide mapping of nodulin 26 phosphorylated in intact nodules. SM proteins from soybean root nodules labeled with ^{32}P-phosphate were digested with Lys-C and were analyzed by TLE as described in Materials and Methods. Autoradiogram of TLE analysis of 500 cpm of (**A**) nodulin 26 phosphopeptides and (**B**) *in vitro*-labeled, Lys-C digested CK-15.

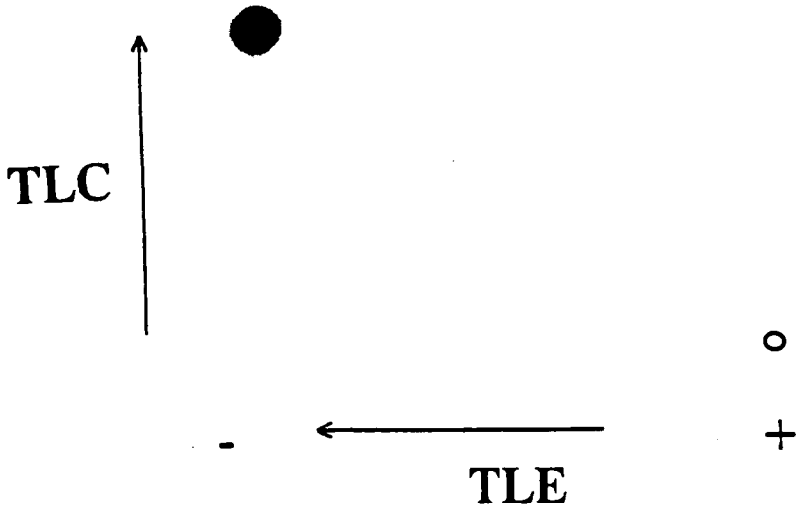


Figure 19. Two-dimensional phosphopeptide mapping of *in vitro*-labeled nodulin 26 and CK-15. 3000 cpm of nodulin 26 and CK-15 Lys-C phosphopeptides were mixed and subjected to two-dimensional analysis (TLE followed by TLC) and autoradiography. O, origin.

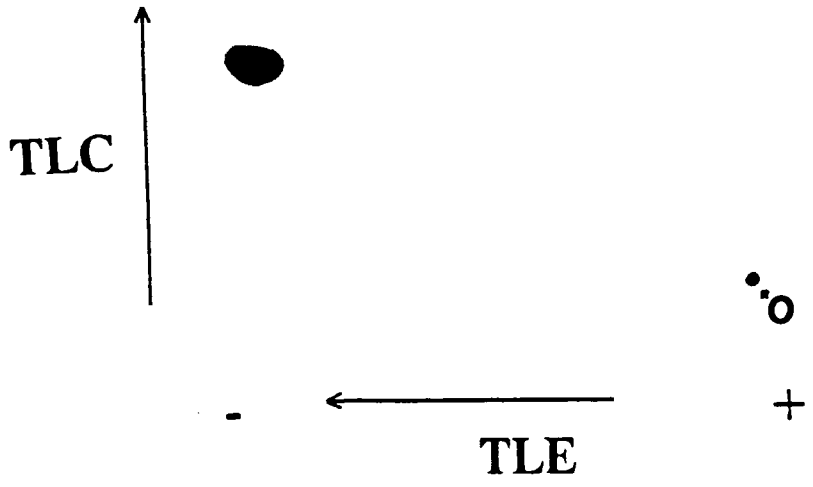


Figure 20. Two-dimensional phosphopeptide mapping of nodulin 26 phosphorylated in intact nodules and *in vitro*-labeled CK-15. 500 cpm each of nodulin 26 Lys-C phosphopeptides and of Lys-C digested, *in vitro*-phosphorylated CK-15 were mixed and subjected to two-dimensional analysis (TLE followed by TLC) and autoradiography. O, origin.

same phosphorylation site.

To confirm that the phosphopeptides were identical, Lys-C phosphopeptides from CK-15 and nodulin 26 were purified by reverse-phase HPLC (Fig. 21). The phosphopeptide from Lys-C digested nodulin 26 eluted as a single peak of radioactivity which corresponded to a major peptide peak, as determined by absorbance at 210 nm (Fig. 21). The Lys-C phosphopeptide from CK-15 eluted from the reverse-phase column with a retention time identical to the nodulin 26 phosphopeptide. Automated Edman degradation of the purified nodulin 26 phosphopeptide yielded the amino acid sequence S-A-S-F-L-K. This corresponds to a unique sequence found in the hydrophilic carboxyl-terminal portion of nodulin 26, as well as in CK-15 (Fig. 5). Furthermore, amino acid composition analysis of the HPLC-purified CK-15 phosphopeptide yielded an amino acid composition that is consistent with this sequence (Table VII). These results strongly support the claim that nodulin 26 and CK-15 possess the same phosphorylation site.

The nodulin 26 and CK-15 Lys-C phosphopeptides each contain two serines. It was not clear from Edman degradation which of these serines was phosphorylated. This uncertainty arises from a technical difficulty associated with automated Edman analysis by the gas-phase sequencer. During Edman degradation, the phosphodiester bond of amino-terminal phosphoserine residues is hydrolyzed. Thus, during the Edman cycle in which the phosphoserine is cleaved from the peptide,

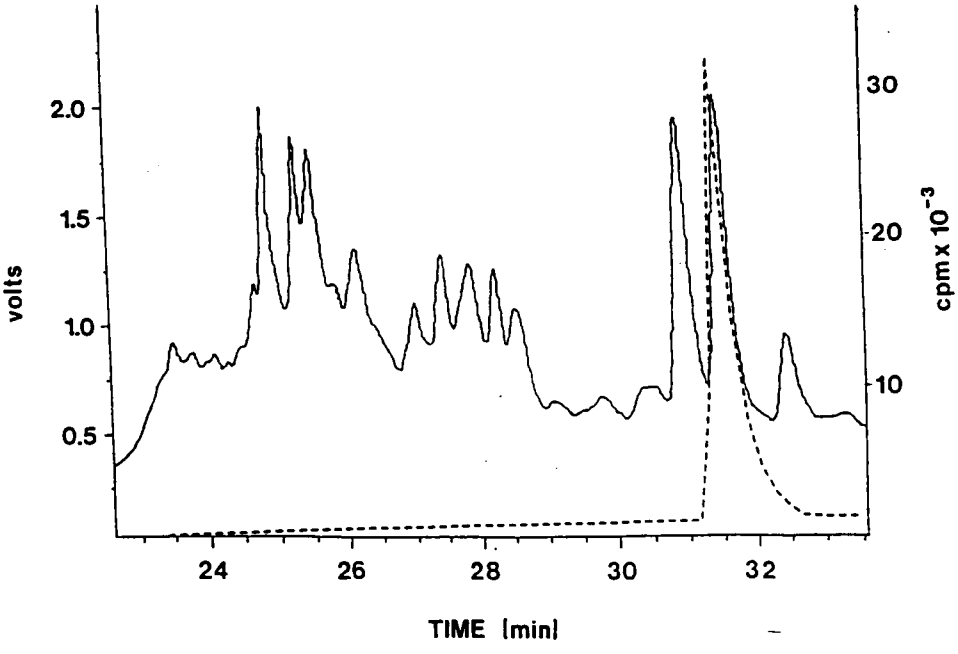


Figure 21. Purification of the major phosphopeptide of nodulin 26. Purified SM were incubated with γ-^{32}P-ATP in the presence of 0.5 mM $CaCl_2$, were digested with Lys-C, and were chromatographed on a C_{18} SEP-PAK cartridge as described in Materials and Methods. C_{18} SEP-PAK fractions containing radioactivity were pooled, and peptides were separated by reverse-phase HPLC using a C_{18} column. Peptides were detected in the eluate by monitoring the absorbance at 210 nm (solid line). Phosphopeptides were detected in the eluate by collecting 100 μl fractions and measuring Cerenkov radiation (dashed line).

Table VII. Amino acid composition of CK-15 Lys-C phosphopeptide[a]

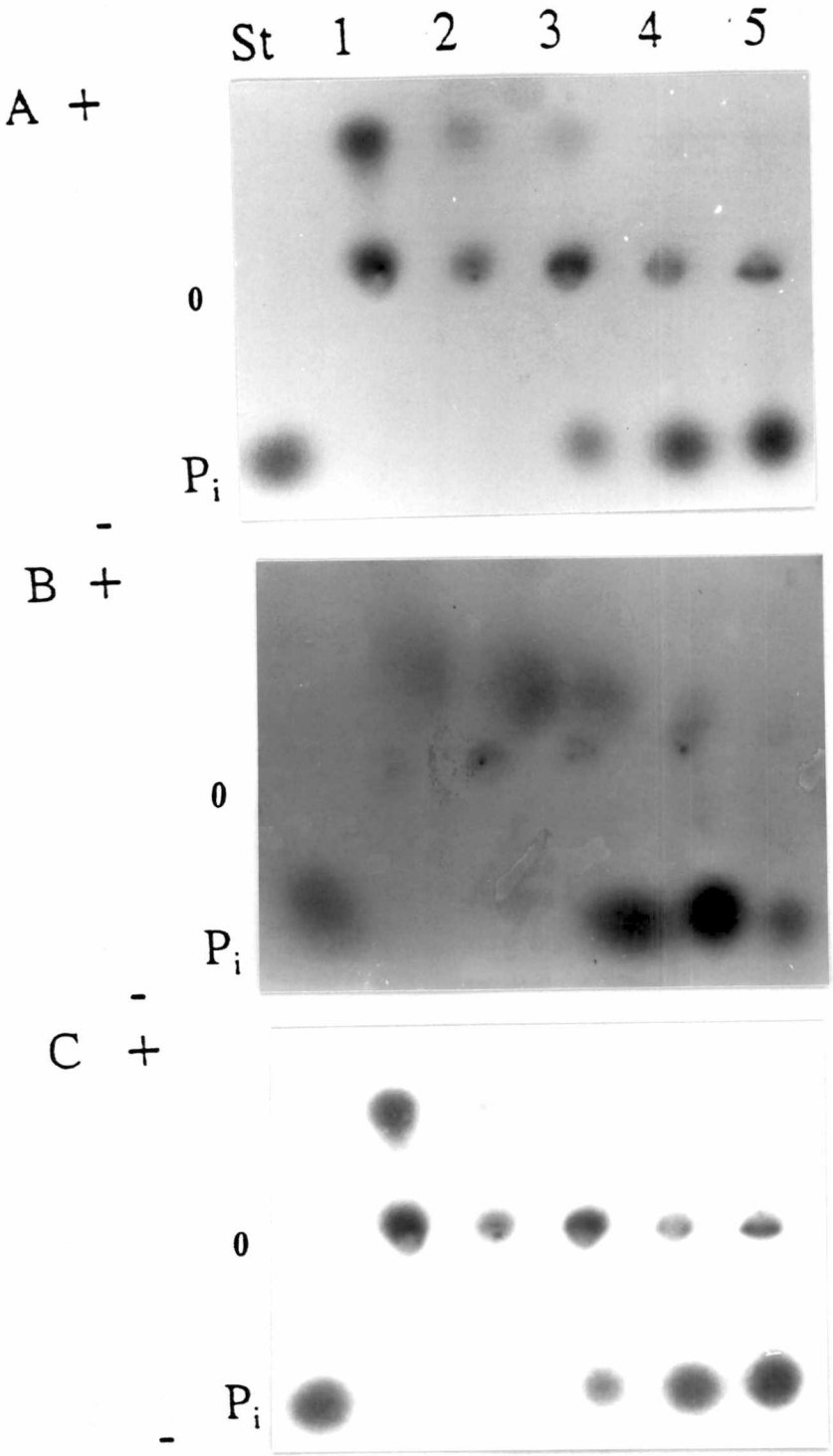
residue	total pmol	mol of residues/mol of peptide
S	622	2.1
A	263	0.9
F	389	1.3
L	353	1.2
K	353	1.2

[a]Results are from the analysis of 300 pmol of isolated CK-15 phosphopeptide. The peptide was purified by reverse-phase HPLC of a Lys-C digest of phosphorylated CK-15 by using the conditions described in Figure 21. Hydrolysis was performed *in vacuo* in 6 N HCL at 110° C for 24 h.

the phosphate is released and is retained on the glass fiber filter (Wang *et al.*, 1988). This precludes positive identification of the phosphorylated residue.

Therefore, the identity of the phosphorylated residue in the Lys-C phosphopeptides from nodulin 26 and CK-15 was determined by manual Edman degradation. This technique allows the identification of the phosphoserine residue by determining the cycle where the ^{32}P-phosphate is released from the peptide (Girault *et al.*, 1989). Manual Edman degradation of all phosphopeptides indicated that phosphate was released during the third cycle which indicates that ser^{262} of nodulin 26 (Fig. 22A & B) and ser^{6} of CK-15 (Fig. 22C) are the sites of phosphorylation. Phosphate is not released during the first cycle which would indicate phosphorylation of ser^{260} in nodulin 26 (Fig. 22A & B) and ser^{4} in CK-15 (Fig. 22C). Taken together, the phosphopeptide mapping, sequence and amino acid composition analyses, and manual Edman degradation analysis indicate that nodulin 26 is phosphorylated both *in vitro* and *in vivo* on a single residue, ser^{262}. These data also demonstrate that CK-15 contains the site in nodulin 26 that is recognized and phosphorylated by the SM-associated CDPK. Interestingly, the serine residues contained in the other R/K-X-X-S motifs in nodulin 26 and CK-15 are not recognized and phosphorylated by the SM-associated CDPK.

Figure 22. Identification of the phosphorylated residue in CK-15 and nodulin 26 by manual Edman degradation. Phosphopeptides were isolated and subjected to manual Edman degradation and were analyzed for the release of ^{32}P-phosphate by TLE as described in Materials and Methods. **A**, the Lys-C phosphopeptide from *in vitro*-labeled nodulin 26. **B**, the Lys-C phosphopeptide from nodulin 26 ^{32}P-labeled in intact nodules. **C**, the Lys-C phosphopeptide from labeled CK-15. St, inorganic phosphate standard (P_i). **O**, origin. Numbers indicate how many cycles of Edman degradation were performed prior to elution and analysis of the cleaved products.


St
1
2
3
4
5
A +
0
Pi
-
B +
0
Pi
-
C +
0
Pi
-

Functional analyses of nodulin 26

As discussed above, nodulin 26 has been proposed to be a member of a family of membrane proteins (Reizer *et al.*,1993). Since transport functions are known for some of these proteins (Heller *et al.*, 1980; Ehring *et al.*, 1990; Preston *et al.*, 1992; van Hoek and Verkman, 1992; Maurel *et al.*, 1993) it has been suggested that nodulin 26 may have a transport function (Sandal & Marcker, 1990). However, direct investigation of the functional properties of nodulin 26 has not been addressed.

Since malate is the major carbon source transported into the symbiosome, we investigated the rate of malate uptake in intact symbiosomes under conditions that favor nodulin 26 phosphorylation or dephosphorylation. Phosphorylation of nodulin 26 on intact symbiosomes was done by incubation in the presence of γ-^{32}P-ATP and Ca^{2+} (Fig. 23). Dephosphorylation of these symbiosomes was accomplished by subsequent treatment with alkaline phosphatase (Fig. 23). When the symbiosomes were re-isolated on Percoll gradients after alkaline phosphatase treatment, nodulin 26 could be phosphorylated again in the presence of γ-^{32}P-ATP and Ca^{2+} (Fig. 23). The kinetics of symbiosome malate uptake were then measured after these treatments. When nodulin 26 was dephosphorylated by alkaline phosphatase, the malate uptake rate was decrease approximately 3-fold compared to phosphorylated symbiosomes (Table VIII). The rate of malate uptake after alkaline

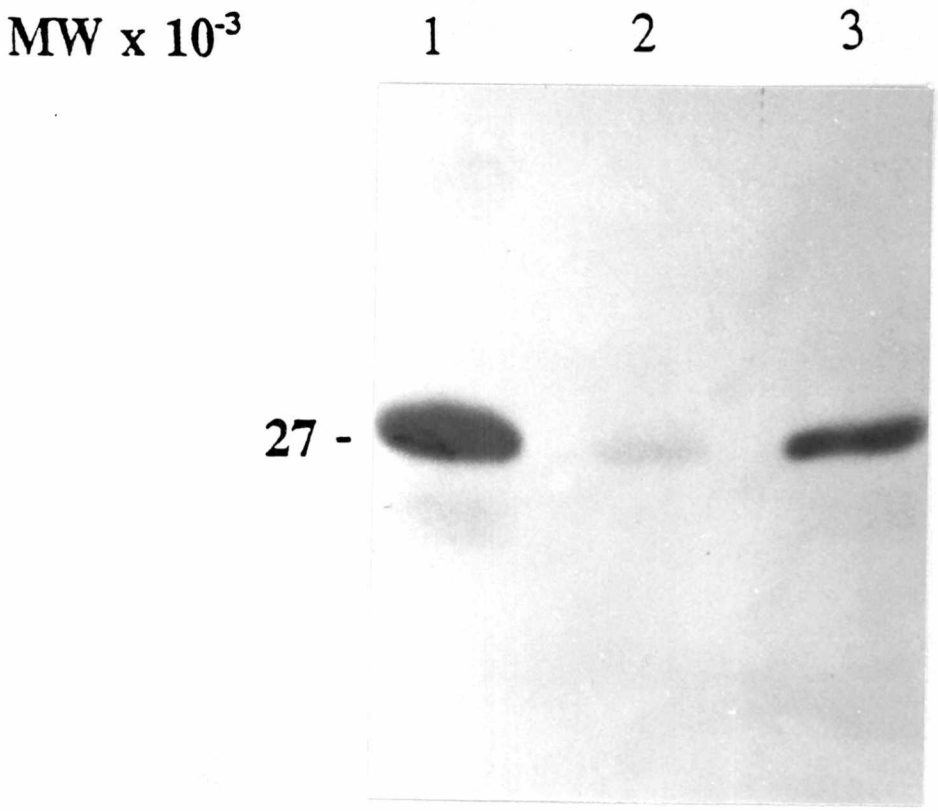


Figure 23. Phosphorylation and dephosphorylation of SM proteins. Isolated symbiosomes were incubated with γ-^{32}P-ATP and Ca^{2+}, or with alkaline phosphatase as described in Materials and Methods. The symbiosomes were subsequently ruptured, and SM were isolated and were subjected to SDS-PAGE, and autoradiography. **1**, symbiosomes treated with γ-^{32}P-ATP and Ca^{2+}. **2**, symbiosomes treated with alkaline phosphatase after incubation with γ-^{32}P-ATP and Ca^{2+}. **3**, symbiosomes incubated with γ-^{32}P-ATP and Ca^{2+} after phosphatase treatment (96 μg total protein/lane). Data taken from Ou Yang *et al.* (1991).

Table VIII. Effect of alkaline phosphatase and Ca^{2+}/ATP treatments on malate uptake by soybean symbiosomes

Assay conditions[a]	Rate of malate uptake[b]	
	Untreated symbiosomes	Phosphatase treated symbiosomes
Control	100	55
+ATP and $CaCl_2$	155	116
+Phosphatase	55	37
+KCN	51	n.d.
+CCCP	48	n.d.

[a] ^{14}C-malate uptake was measured for 1 min as described in Materials and Methods, using 0.3 mM malate as substrate. Where indicated, symbiosomes were preincubated (10 min) with 1 mM ATP, 0.5 mM $CaCl_2$; with 48 U of alkaline phosphatase; or with 0.5 mM KCN, or 5 μM CCCP. Symbiosomes were washed on Percoll gradients after incubation, to remove alkaline phosphatase.

[b] Rates are expressed as percentages of the control rate (7.7 nmol/min/mg protein) obtained with untreated symbiosomes. n.d., not determined. Data taken from Ouyang *et al.* (1991).

phosphatase treatment was comparable to the rates observed after treatment with the potent inhibitors of symbiosome malate uptake, KCN and CCCP (Table VIII). The decrease in malate uptake observed after treatment with alkaline phosphatase could be substantially reversed by incubating symbiosomes under conditions that favored the rephosphorylation of nodulin 26 (Table VIII). These data suggested a correlation between the phosphorylation state of nodulin 26 and SM-malate transport. Because of the proposed role of nodulin 26 as a transport protein, it is attractive to consider the possibility that nodulin 26 is responsible for malate transport. However, these experiments alone are not adequate evidence that nodulin 26 is the protein mediating the transport of malate across the SM.

To investigate the functional properties of nodulin 26 directly, the protein was purified in an undenatured form. Purified SM were used as a starting material for the purification of nodulin 26 since it is a major protein in this membrane fraction (Fig. 24A). Prior to nodulin 26 solubilization, SM were washed with a buffer containing 1 M KI. This procedure also has been used to remove extrinsic membrane proteins prior to the solubilization of another MIP family member, CHIP, from erythrocyte membranes (Smith & Agre, 1991). Nodulin 26 was solubilized from the KI-washed SM using the non-ionic detergent OG. This protocol yielded near quantitative extraction of nodulin 26. The solubilized SM

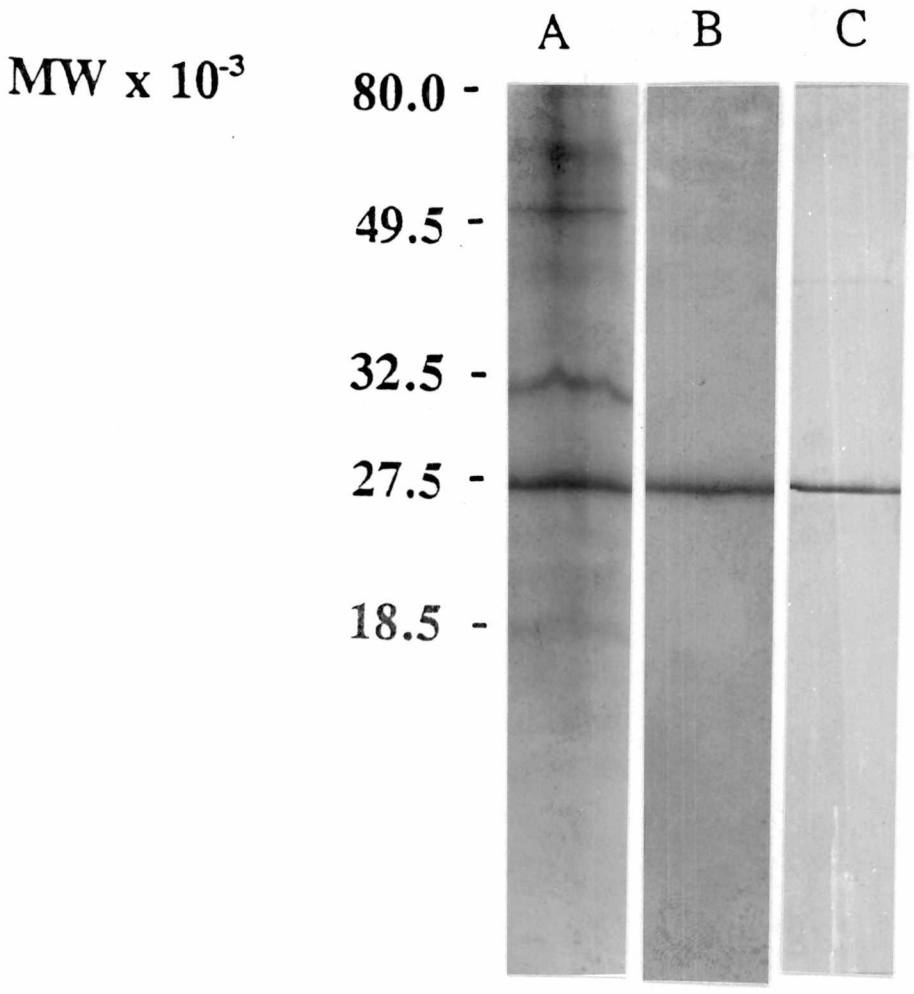


Figure 24. SDS-PAGE analysis of purified nodulin 26. SDS-PAGE was done on 15 % (w/v) polyacrylamide gels **A**, purified SM (20 μg total protein); **B**, purified, concentrated nodulin 26 (5.0 μg total protein); **C**, purified nodulin 26 (0.5 μg total protein) blotted onto nitrocellulose and probed with anti-CK-15 antibody. The proteins in lanes A and B were stained with Coomassie Blue.

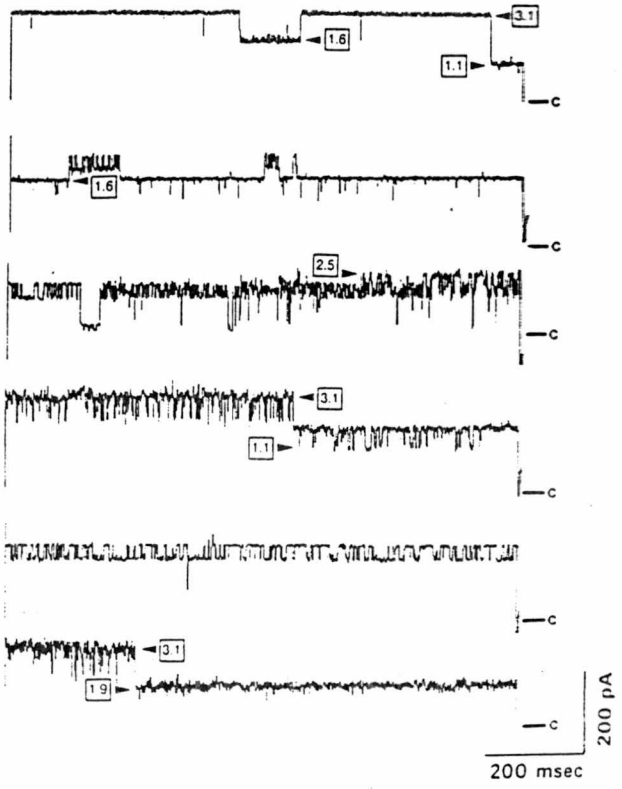
proteins were chromatographed on a DEAE Fast Flow column attached to a FPLC. Nodulin 26 was eluted with a linear NaCl gradient. Fractions from the DEAE Fast Flow column were examined by SDS-PAGE and immunoblotting using anti-CK-15 antibodies. The results of the purification revealed a single Coomassie blue-stained band that migrated with an apparent molecular weight of 27,000 (Fig. 24B). The identity of this band as nodulin 26 was confirmed by Western blot with anti-CK-15 antibodies (Fig. 24C).

The successful purification of nodulin 26 under nondenaturing conditions allowed for direct assay of its functional activity using planar lipid bilayer techniques. This technique has been used successfully to investigate the ion channel properties of a number of proteins (Labarca and Latorre, 1992) including the nodulin 26 homolog MIP (Ehring *et al.*, 1990; 1991). Nodulin 26 was prepared for incorporation into planar lipid bilayers by reconstitution into small unilamellar phosphatidylcholine liposomes. The presence of nodulin 26 in the liposomal membrane was verified by Western blot analysis.

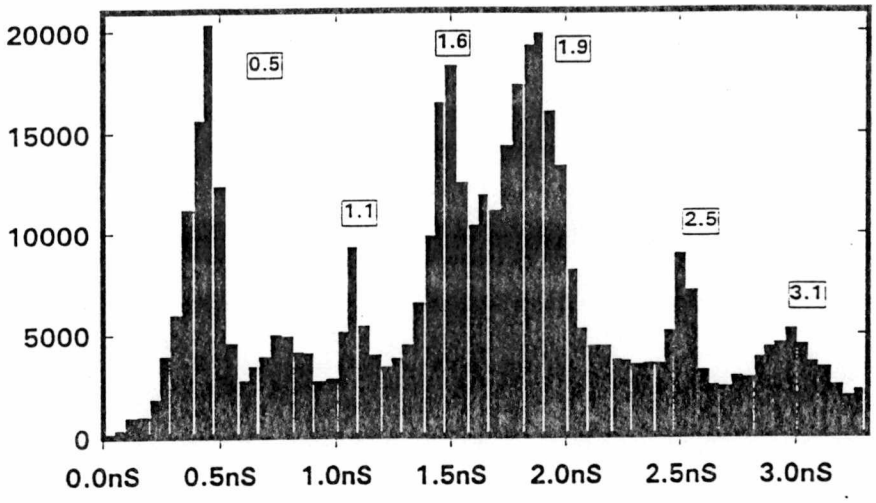
Channel activity was assayed by allowing liposomes containing nodulin 26 to fuse with planar lipid bilayers painted across a septum in a bilayer chamber. Addition of nodulin 26 to the planar lipid bilayers resulted in the appearance of ion-conducting channels, usually within 30 min after first addition (Fig. 25). However, channels were not

Figure 25. Single channel recordings of purified, reconstituted nodulin 26. All channel recordings were done in a recording solution of 1 M KCl, 20 mM MOPS-NaOH, pH 7.4, as described in Materials and Methods. **A**, Representative channel traces. The holding potential was 70 mV. The channel openings are shown as upward deflections. **c-** on the right side of the traces indicates the closed (zero-current) position. Arrows indicate conductance levels corresponding to the labeled conductance amplitude histogram peaks in panel B. **B**, Amplitude histogram for nodulin 26 channels shown in panel A with principal conductance states of 0.5, 1.1, 1.6, 1.9, 2.5, and 3.1 nS. The ordinate represents the total number of events recorded. The events were not weighted according to the duration of the conductance state.

A


3.1
1.6
1.1
c
1.6
c
2.5
c
3.1
1.1
c
c
3.1
1.9
c
200 pA
200 msec

B


20000
15000
10000
5000
0
0.5
1.1
1.6
1.9
2.5
3.1
0.0nS
0.5nS
1.0nS
1.5nS
2.0nS
2.5nS
3.0nS

observed in experiments where nodulin 26-free phosphatidylcholine liposomes were added to the bilayer chamber, even after 3 h of incubation. This suggests that the channels observed following the addition of nodulin 26 proteoliposomes were due to the insertion of nodulin 26 into the bilayer.

Out twelve channels evaluated by this method, ten apparently were due to the incorporation of single nodulin 26 channels. This proposal is based on the fact that these channels displayed a maximum conductance state of 3.1 nS (Fig. 25B) and showed similar lower conductance states. This conductance was derived from a current-voltage plot of the maximum current amplitude at each voltage (Fig. 26). The other two recordings showed much higher conductance states and probably resulted from the incorporation of multiple channels into the bilayer.

Multiple conductance states are apparent in the single channel records of nodulin 26 channels (Fig. 25). Current-voltage relationships for the 3.1 nS and the 1.6 nS conductance states were linear over the range of -100 mV to +100 mV, and the measured reversal potential was near zero under standard conditions (Fig. 26). Additional conductance states were identified from current-amplitude histograms determined at several different voltages. The conductance of each peak was calculated from the current by assuming a linear relationship (conductance = current/voltage). The most

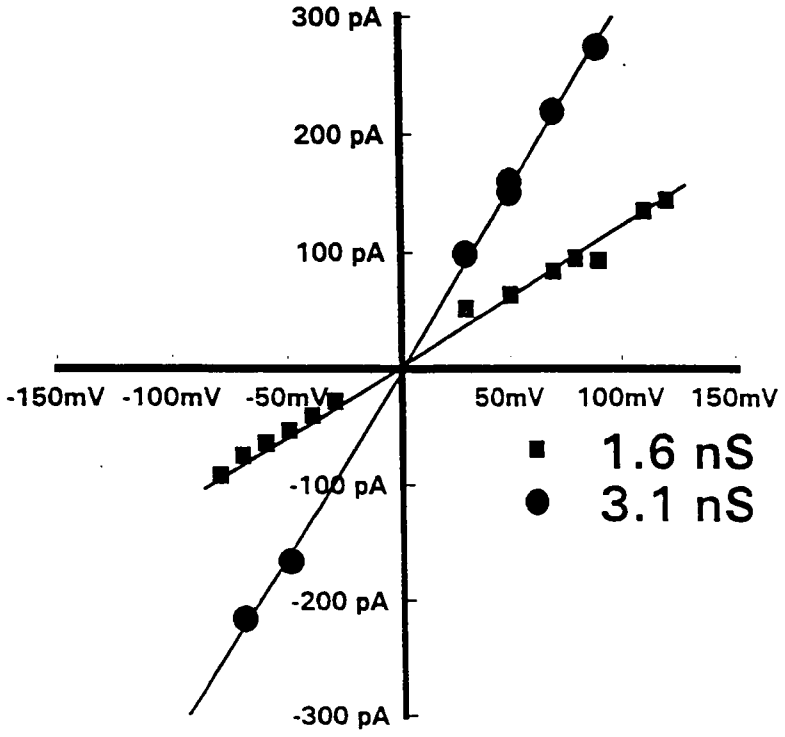


Figure 26. Current-voltage relationships of the 3.1 nS and 1.6 nS conductance states of nodulin 26. Channel recordings were done in a recording solution of 1 M KCl, 20 mM MOPS-NaOH, pH 7.4, as described in Materials and Methods. Single current amplitudes were determined from pulsed potentials to the indicated voltage, as described in Materials and Methods.

frequently observed states had conductances of 0.5 nS, 1.1 nS, 1.6 nS, 1.9 nS, 2.5 nS, and 3.1 nS (Fig. 25B) and were observed in at least five of the ten nodulin 26 channel records examined.

The channel records showed widely variable open dwell times that ranged from greater than 50 msec to 1 msec (Fig. 25). Under the experimental conditions, channel closures to the bare membrane conductance were rare, and were more often observed at higher voltages (60-100 mV) than at lower voltages (<40 mV). Channels typically exhibited transitions between the various open conductance states, ant the occupancy of these various states appeared to be voltage dependent (Fig. 27).

The occupancy times of the various conductance states showed a dependence on voltage (Fig. 27). Figure 27 shows channel traces and conductance amplitude histograms of a single nodulin 26 channel recorded at 30 and 70 mV. In this figure, the amplitude histogram events are weighted to indicate the time of occupancy of the different conductance levels (this contrasts with figure 25B where total events are shown with no weighting by duration). At 30 mV, the channel occupied the 3.1 nS state 76% of the time, with infrequent transitions to the lower conductance states. In contrast, at 70 mV the channel occupied the 3.1 nS state only 7.2% of the time, and greater occupancy of the lower conductance states was observed at this potential. These data indicate that

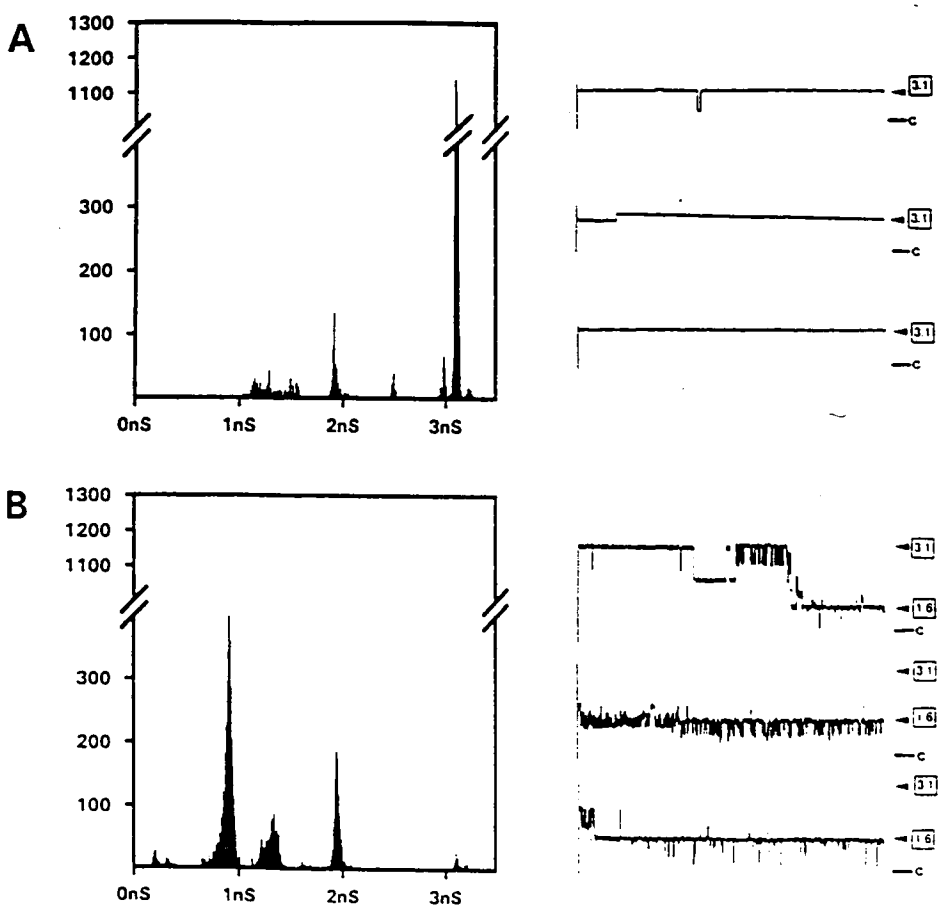


Figure 27. Voltage sensitivity of nodulin 26 ion channels. Nodulin 26 channel conductance amplitude histograms corresponding to channels recorded in symmetric 1 M KCl, 20 mM MOPS-NaOH, pH 7.4 at a potential of (**A**) 30 mV and (**B**) 70 mV are shown. Data were derived from 25 one-second sweeps similar to those shown to the right of each histogram. The ordinate represents the number of events recorded. The TRANSIT program was used to weight the events by the duration of the occupancy of the indicated conductance states.

nodulin 26 forms a voltage-sensitive channel with lower conductance states preferentially occupied at higher voltage potentials.

The ion selectivity of the nodulin 26 channel was investigated with respect to the charge and size of the ions that were conducted. To do this, asymmetric conditions were established with respect to the size and concentration of ions in the *cis* and *trans* chambers of the planar lipid bilayer apparatus. Under these conditions, the reversal potential was measured to determine which ions the channel preferred to conduct. In experiments in which the *trans* chamber solution was changed from 1 M KCl, 20 mM MOPS, pH 7.4 to 0.1 mM KCl, 20 mM MOPS, pH 7.4, a small shift in the reversal potential was observed (-4 mV) (Fig. 28). This -4 mV shift in reversal potential corresponds to a permeability ratio of (PK^+:PCl^-) 1:1.21. Thus, there is only a slight selectivity for Cl^- under these conditions. In an attempt to discern whether the nodulin 26 channel could discriminate between small and large ions, the *trans* chamber solution was changed to 1 M Tris-HEPES, pH 7.4. The maximum conductance decreased from 3.1 nS to 0.5 nS at negative voltage potentials (i.e., *trans* side conductance) and from 3.1 nS to 1 nS at positive voltage potentials (i.e., *cis* side conductance) (Fig. 29). This effect suggests that large ions may block the full opening of the nodulin 26 channel. In addition, the finding that the *trans* side conductance (which represents the current of $Tris^+$

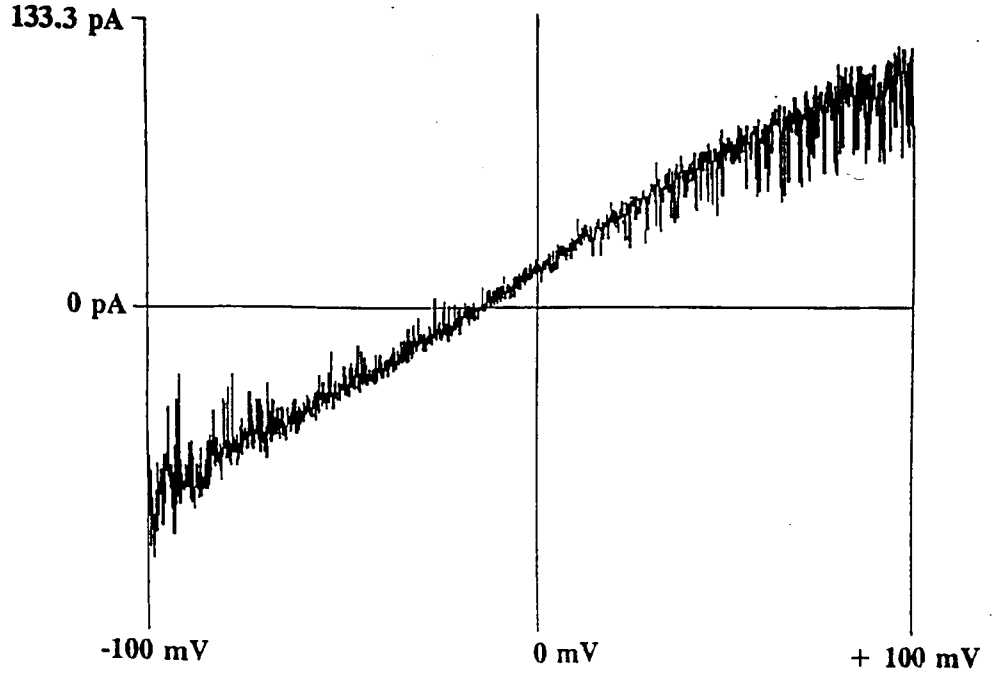


Figure 28. Current-voltage trace of the nodulin 26 ion channel in asymmetric KCl solutions. A representative trace of a nodulin 26 channel recorded with the ramp protocol described in Materials and Methods is shown. The recording solution was 1 M KCl, 20 mM MOPS-NaOH, pH 7.4 (*cis*) and 0.1 M KCl, 20 mM MOPS, pH 7.4 (*trans*).

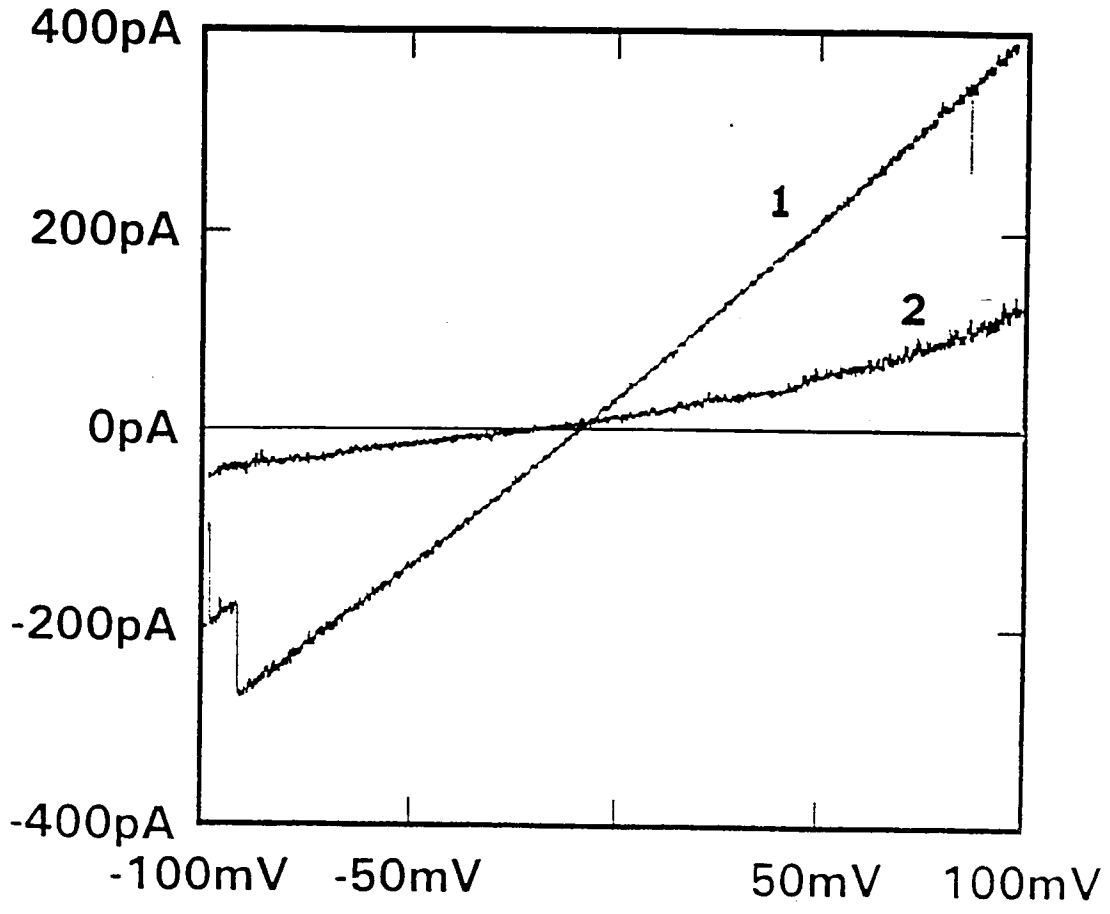


Figure 29. Current-voltage traces of the nodulin 26 ion channel in Tris-HEPES. Representative traces of a nodulin 26 channel recorded with the ramp protocol described in Materials and Methods are shown. The recording solution was symmetrical 1 M KCl, 20 mM MOPS-NaOH, pH 7.4 (Trace **1**), or *cis* 1 M KCl, 20 mM MOPS-NaOH, pH 7.4, *trans* 1 M Tris-HEPES, pH 7.4 (Trace **2**).

from the *trans* side to the *cis* side) is decreased more than the *cis* side conductance suggests that $Tris^+$ is a more effective blocker of the nodulin 26 channel than $HEPES^-$. Therefore, it appears that the nodulin 26 channel is weakly anion selective and that it prefers to transport the smaller K^+ and Cl^- ions compared to the larger $Tris^+$ and $HEPES^-$.

The observation that nodulin 26 phosphorylation was correlated with increased malate transport in intact symbiosomes raised the question of whether the nodulin 26 channel was capable of transporting malate. This was addressed by planar lipid bilayer measurements with 0.1 M potassium malate, 20 mM MOPS-NaOH, pH 7.4 in the *trans* chamber, and 1 M KCl, 20 mM MOPS-NaOH, pH 7.4 in the *cis* chamber. In experiments using the ramp protocol described in Materials and Methods, no significant change in the reversal potential was observed (Fig. 30) suggesting that the nodulin 26 channel is capable of transporting malate under these conditions.

To address the functional effects of nodulin 26 phosphorylation, alkaline phosphatase was added to both the *cis* and *trans* bilayer chambers during the course of recording a nodulin 26 channel under standard conditions at -70 mV. Alkaline phosphatase treatment of the nodulin 26 channel resulted in opening to higher conductance states more frequently compared to the untreated channel (Fig. 31). While preliminary, these data suggest that the dephosphorylated

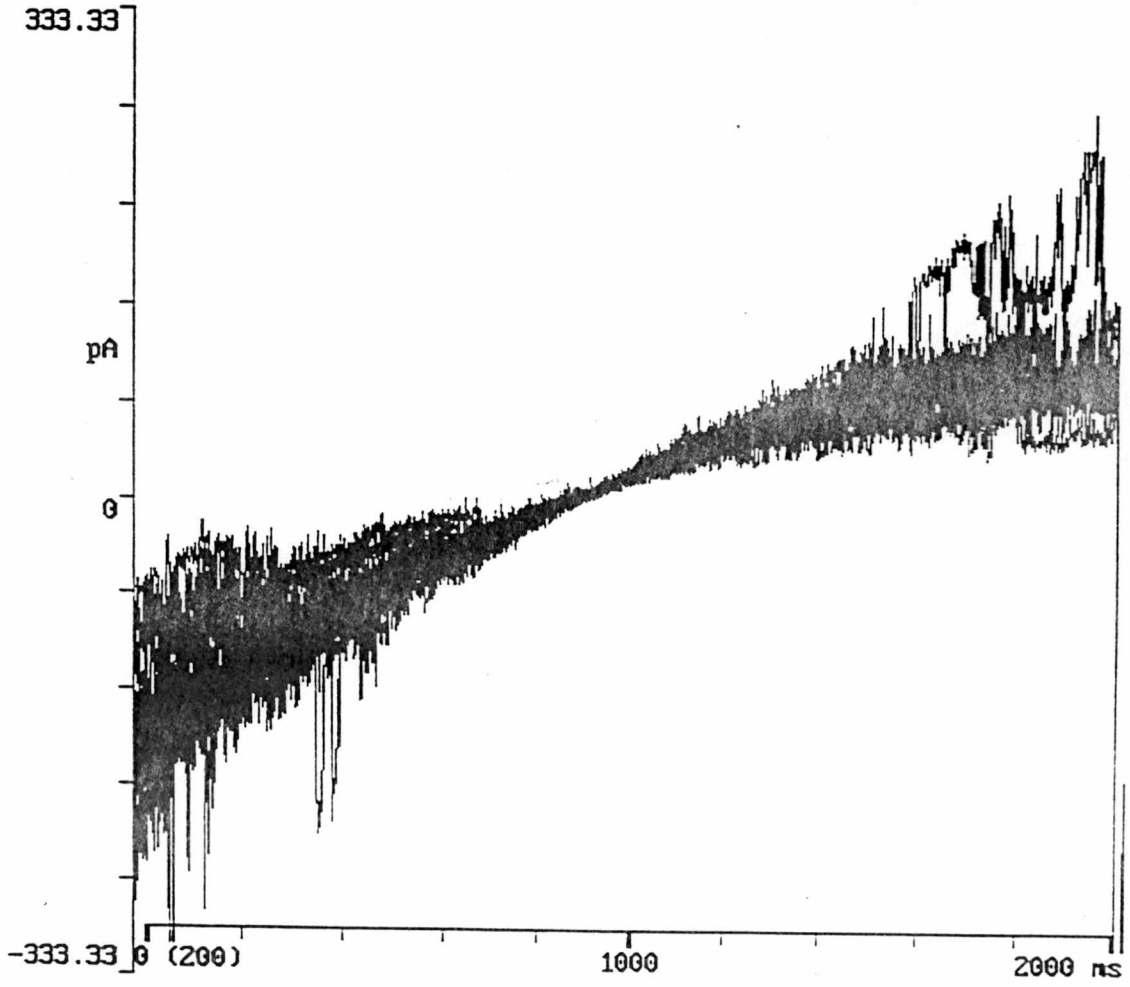


Figure 30. Current-voltage trace of the nodulin 26 ion channel in potassium malate. Representative trace of a nodulin 26 channel recorded with the ramp protocol described in Material and Methods is shown. The recording solution was 1 M KCl, 20 mM MOPS-NaOH, pH 7.4 (*cis*) and 0.1 M potassium malate, 20 mM MOPS-NaOH, pH 7.4 (*trans*).

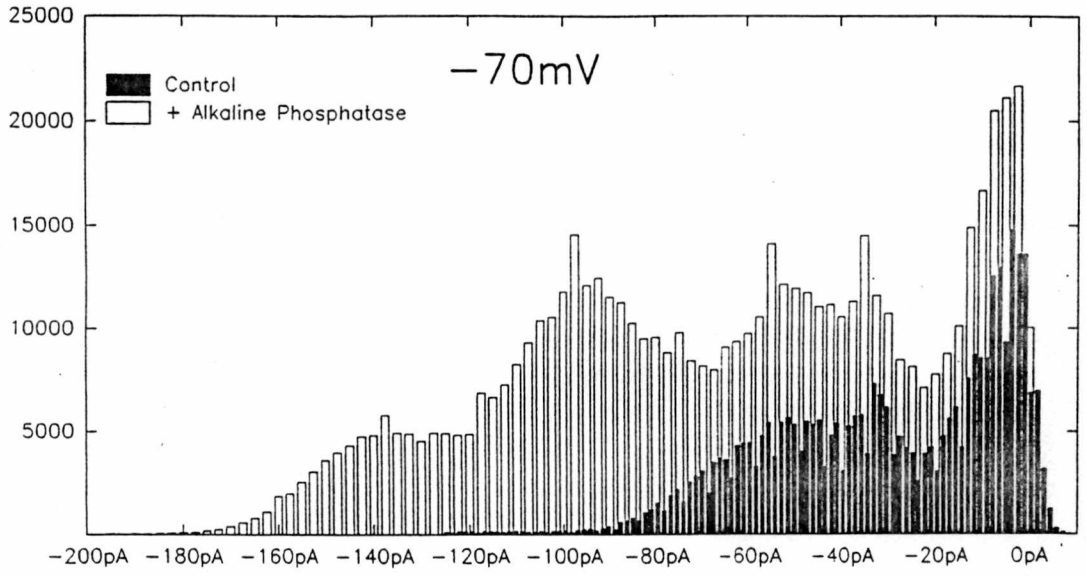


Figure 31. Amplitude histogram of a nodulin 26 ion channel before and after treatment with alkaline phosphatase. Recordings were made using the pulsing protocol described in Materials and Methods. The recording solution was 1 M KCl, 20 mM MOPS-NaOH, pH 7.4. Filled bars, control (untreated) nodulin 26 channel; open bars, the same nodulin 26 channel incubated with 15 units of alkaline phosphatase, added to both the cis and trans sides of the bilayer chamber. The ordinate represents the number of events recorded. The events were not weighted according to the duration of the conductance state. The abscissa represents the channel current measured in pA.

nodulin 26 channel may be less sensitive to voltage-dependent channel closure.

CHAPTER IV

DISCUSSION

Nodulin 26 localization and expression

Previous investigations suggested that nodulin 26 was a nodule-specific protein and that it was localized to the SM (Fortin *et al.*, 1987). Nodule-specific expression of nodulin 26 was based on the observation that nodulin 26 cDNA was obtained from a library which was depleted of clones encoding proteins from uninfected roots. However, this approach was only based on differential cDNA screening of nodule and root libraries and did not take into consideration the potential for nodulin 26 expression in other tissues (Fortin *et al.*, 1987). The localization of nodulin 26 to the SM was inferred by the observation that the cDNA probe used to isolate the nodulin 26 clone from the nodule cDNA library was obtained from mRNA associated with polysomes immunoprecipitated with anti-SM antibodies (Fortin *et al.*, 1987). In addition, the *in vitro* translation product of the nodulin 26-mRNA (obtained by hybrid selection with the nodulin 26 cDNA) was immunoprecipitated by anti-SM antibodies (Fortin *et al.*, 1987). Overall, these data indicated that the nodulin 26 gene is expressed in nodules and not in roots, and that nodulin 26 might be found in the SM. However, these data did not establish that nodulin 26 was localized exclusively to the SM since the method used did not address whether nodulin 26 was present in other membrane fractions.

These problems were addressed in the present study by using immunolocalization techniques to detect and localize nodulin 26 protein in nitrogen-fixing soybean root nodules. Nodulin 26-specific antibodies were prepared by using two antigens: 1. a synthetic peptide corresponding to a carboxyl-terminal sequence unique to nodulin 26 (CK-15)(Weaver *et al.*, 1991), or 2. SDS-PAGE purified nodulin 26. Nodulin 26 was identified by Western blots with anti-CK-15 antibodies as a major protein in purified SM that migrated with an apparent MW of 27,000 (Weaver *et al.*, 1991). Immunoelectron micrographs, made by using anti-nodulin 26 antisera to probe nodule sections, clearly demonstrated that nodulin 26 protein is exclusively localized to the SM. Immunoreactivity was not observed with other membrane systems, including the plasma membrane and the tonoplast, in mature, nitrogen-fixing root nodules. These data positively establish that nodulin 26 is a SM-specific protein in the root nodule.

In order to investigate more thoroughly the nodule specificity of nodulin 26 protein expression, anti-CK-15 antibodies were used to probe membrane samples from other tissues of soybean. This tissue survey clearly showed that nodulin 26 protein is only detected in root nodules and not in roots, stems, leaves, or apices. Recently Miao & Verma (1993) have used a gene construct, containing the promoter region of the nodulin 26 gene coupled to a reporter gene, to address the tissue specificity of the nodulin 26 promoter activity in

transgenic soybean roots. The results of their study corroborate our data, indicating that the nodulin 26 promoter is only active in root nodules in soybeans. Taken together, these data establish the tissue specificity of nodulin 26 expression in soybean.

As discussed in the introduction, the timing of expression of nodulin genes has been divided into two broad classes, the early nodulins (ENODs) and the late nodulins (NODs) (reviewed in Franssen *et al.*, 1992). ENODs are expressed during the pre-infection, infection, and nodule organogenesis stages, whereas the NODs are expressed at or near the onset of nitrogen fixation. Previously, it has been reported that the nodulin 26 gene is expressed in nodules that result from the infection of certain mutant strains of bacteria (Fortin *et al.* 1987; Morrison & Verma, 1987). These mutants are not released from their infection threads into symbiosomes (bar$^-$ mutants) and therefore are unable to fix nitrogen. In the present study, an attempt was made to determine the levels of expression of nodulin 26 protein in nodules caused by the infection of soybean by bar$^-$ mutant rhizobia. Although the bacteria are not released into symbiosomes, vast quantities of membrane bound structures that fill the cytoplasm of plant cells that would normally be the target of rhizobium infection (Roth & Stacey, 1989). It has been suggested that these vesicles may be empty symbiosomes (Regensburger *et al.*, 1986) or some type of degradative

vesicles (Roth & Stacey, 1989). A comparison of anti-CK-15 immunoreactivity in membrane fractions from nodules that arise from the infection of wild-type and mutant bacterial strains revealed that there was not a significant difference in the amount of nodulin 26 protein found in these membranes.

These data suggest that not only is the nodulin 26 gene expressed and translated, but also that steady-state nodulin 26 protein levels are relatively unaffected by the failure of bacteria to be released into the symbiosome or by the lack of onset of nitrogen fixation. Currently it is not known where nodulin 26 is expressed in these mutant nodules. It may be targeted to the vesicles that fill the cytoplasm of the mutant nodules cells by default if there is no SM. Therefore, immunoelectron microscopy needs to be done, using anti-nodulin 26 antibodies, to localize nodulin 26 in these ineffective nodules.

In contrast to nodulin 26, the SM nodulin 24 is only expressed when bacteria are released into the symbiosome (Fortin *et al.*, 1987). This suggests that there is differential regulation of the expression of SM nodulins, which is supported by the fact that the nodulin 26 promoter differs in structure from those observed for many other nodulins, including nodulin 24 (reviewed in Franssen *et al.*, 1992; Miao & Verma, 1993). Thus, the division of late nodulins into one broad developmental class may be an oversimplification.

Since the physiological functions of nodulin 26 and nodulin 24 remain unknown, it is not clear why differential regulation might be necessary. The observation that nodulin 26 is expressed in mutant nodules that make infection threads but not in nodules that lack infection threads (Morrison & Verma, 1987), has led to the suggestion that nodulin 26 may possess a role that begins prior to release of the bacteria into the SM (Nap and Bisseling, 1990). Further work is necessary to identify a role for nodulin 26 during these early nodule developmental processes.

Detection, purification, and characterization of CDPK

The deduced amino acid sequence of nodulin 26 revealed that the molecule contains five potential phosphorylation sites with the sequence R/K-X-X-S. This sequence is characteristic of the phosphorylation site motif recognized by CDPKs from plants (Roberts & Harmon, 1992). Therefore, this raised the possibility that nodulin 26 might be a target of Ca^{2+}-dependent phosphorylation in soybean root nodules. Since CK-15 contains two of the potential phosphorylation sites in nodulin 26, it was used as a substrate to detect a Ca^{2+}-dependent protein kinase in root nodules. CK-15 was found to be a good substrate for a Ca^{2+}-stimulated protein kinase present in both soluble and particulate fractions from soybean root nodules (Weaver *et al.*, 1991).

The soluble form of this kinase was purified and was found to have a subunit molecular weight of 60,000. This

kinase was stimulated by μM concentrations of Ca^{2+} under physiological conditions of [Mg^{2+}] and pH (Weaver *et al.*, 1991). This level of sensitivity to Ca^{2+} suggests that this kinase would make a suitable sensor for physiologically relevant changes in cytosolic Ca^{2+} concentration. The purified kinase was demonstrated to be free of calmodulin, and it did not require exogenous calmodulin for Ca^{2+} stimulation (Weaver *et al.*, 1991). The kinase was also unaffected by the lipid effectors of Ca^{2+}-dependent protein kinase C (Weaver *et al.*, 1991). In this regard this protein kinase differed from the two most prevalent classes of Ca^{2+}-dependent protein kinases from animals, calmodulin-dependent protein kinases and protein kinase C.

These properties are characteristic of the properties of a family of Ca^{2+}-dependent protein kinase activities (CDPKs) that have been reported throughout the plant kingdom (reviewed in Roberts & Harmon, 1992; Roberts, 1993). The deduced amino acid sequence of CDPKs from soybean tissue culture cells (Harper *et al.*, 1991), *Arabidopsis* (Harper *et al.*, 1993), and carrot (Suen and Choi, 1991) revealed that they possess a calmodulin-like regulatory domain which contains four EF-hand domains, thus allowing direct Ca^{2+} stimulation (reviewed in Roberts and Harmon, 1992 and Roberts, 1993). The Ca^{2+}-stimulation data combined with the observation that CDPK-specific monoclonal antibodies (Putnam-Evans *et al.* 1990)

recognized the soluble Ca^{2+}-dependent CK-15 kinase strongly suggest that this kinase belongs to the CDPK family.

Since the Ca^{2+}-dependent CK-15 kinase activity was detected in both soluble and particulate nodule fractions, including the SM, we attempted to determine whether or not the SM-associated kinase was integrally associated with the membrane. Our results indicated that the kinase could not be dissociated from the membrane by various chaotropic agents and dilute NaOH, treatments commonly used to remove peripherally-associated membrane proteins. However, the kinase could be extracted from the SM by detergent. These results suggested that the SM-associated CDPK interacts with the SM through a hydrophobic domain of the protein or through a posttranslationally-attached membrane anchor.

The existence of this type of membrane-associated CDPK is supported by the fact that membrane-associated CDPKs have been reported in a number of plant systems (Schaller & Sussman, 1988; Klucis & Polya, 1988; Schaller *et al.* 1992). In the case of the enzyme from oat, it has been suggested that soluble CDPK activities arise from limited proteolysis of membrane-associated CDPK activity (Schaller *et al.*, 1992). This is based on data that showed that oat plasma membranes contained two immunologically related proteins recognized by anti-CDPK monoclonal antibodies. These proteins could be converted *in vitro* to a soluble form by limited proteolysis with trypsin. The product of this proteolysis migrated with

the same apparent molecular weight as the immunologically-related soluble form of the kinase from oat. The authors admit that none of the deduced amino acid sequences of the cloned CDPKs reveal a domain that would serve as a transmembrane region or as a membrane anchor. Furthermore, it has yet to be established that any of the membrane-associated CDPKs contain a posttranslationally-attached membrane anchor. Thus, the mechanism of attachment of the membrane-associated CDPKs remains to be established.

In trials where equal amounts of soluble nodule and SM-associated protein kinase activity were separated by SDS-PAGE and probed with anti-CDPK antibodies, immunoreactivity was only observed for the soluble kinase. This is in contrast to the report that the intensity of immunostaining with these antibodies showed a good correlation with the amount of plasma membrane-associated kinase activity from oat on Western blots (Schaller *et al.*, 1992). Assuming a similar k_{cat} for the soluble and SM-associated kinases, these data suggest that they may be different proteins, and that the soluble form is not simply a proteolytic product of the membrane-associated enzyme.

In further support of the possibility that the soluble and membrane-associated CDPKs from soybean may be different proteins, there appear to be multiple isoforms of CDPK. This is based on the observation of differential immunoreactivity of CDPKs with four different anti-soybean tissue culture cell

CDPK monoclonal antibodies (Alice Harmon, personal communication). Also, protein sequence and cDNA sequence data of CDPKs support the existence of isozymes in soybean (Harper *et al.*, 1991) and *Arabidopsis thaliana* (Harper *et al.*, 1993).

A more extensive investigation needs to be performed to characterize the relationship between the soluble and SM-associated CDPK from soybean. At present the kinetic and regulatory properties of the SM-associated CDPK are poorly characterized. Studies of Ca^{2+} sensitivity and lipid stimulation similar to these done on the plasma membrane-associated enzyme from oat (Schaller & Sussman, 1988; Schaller *et al.*, 1992) need to be done. In addition, the cloning and comparison of the sequences of the cDNA encoding soluble and SM-associated CDPKs needs to be undertaken. Hopefully, such an investigation would shed some light on how membrane-associated CDPKs interact with the membrane, and clearly establish that the soluble and membrane-associated activities are associated with different proteins.

Nodulin 26 phosphorylation

The observation that CDPK activity has been found in all plant tissues tested and that it is widely distributed throughout the plant kingdom makes this family of protein kinases a likely candidate as a multifunctional target of intracellular Ca^{2+} signals. The identification of CDPKs role in Ca^{2+}-signalling requires the identification of its endogenous substrates. With the exception of the results

presented in this report, only the H^+-pumping ATPase from oat (Schaller & Sussman, 1988) and α-TIP (Johnson & Chrispeels, 1992) have been identified as endogenous CDPK substrates. The initial observation that CK-15 was a substrate for soybean nodule CDPKs suggested that nodulin 26 might be an endogenous CDPK substrate. This was substantiated by the finding that nodulin 26 was a CDPK substrate *in vitro* and *in vivo*. In fact, it was the only detectable SM protein phosphorylated by the SM-associated CDPK. Thus, this represents one of the few endogenous CDPK substrates identified and provided an opportunity to study the determinants for CDPK recognition and phosphorylation.

It has been previously determined that protein kinase substrate recognition is mediated at least in part by the amino acid residues that closely flank the phosphorylation site in the primary amino acid sequence. Using these observations, phosphorylation site recognition motifs have been established for a number of protein kinases (reviewed in Kemp & Pearson, 1990). It has been observed that CDPKs phosphorylate a large number of exogenous substrates with varying catalytic rates (reviewed Roberts & Harmon, 1992). Based on the observation that many CDPK activities phosphorylate peptides containing the sequence R/K-X-X-S (Roberts, 1989; Polya *et al.*, 1989; Putnam-Evans *et al.*, 1990; Weaver *et al.*, 1991), it has been suggested that this motif may be important for substrate recognition. However,

synthetic peptides containing the sequence S-X-K are also substrates of this protein kinase (Polya *et al.*, 1989). The S-F-K motif was also observed in the phosphorylation site on histone H1 phosphorylated by a Ca^{2+}-dependent protein kinase from alfalfa (Olah *et al.*, 1989) and wheat embryo (Polya *et al.*, 1989).

Since so few endogenous substrates have been identified, very little is known about the structural determinants for CDPK recognition. Recently, the site on α-TIP that is phosphorylated by a tonoplast-associated CDPK has been determined as ser^7 (Johnson & Chrispeels, 1992). The primary amino acid sequence surrounding this site is A-T-R-R-Y-S^{PO4}-F-G-R-T (Johnson & Chrispeels, 1992). It was suggested that the R-R-Y-S sequence agreed with the R/K-X-X-S motif (Johnson & Chrispeels, 1992). However, R-R-Y-S is more characteristic of the phosphorylation site motif of cyclic AMP-dependent protein kinase (X-R-R-X-S-X) (Kemp & Pearson, 1990). Peptides and proteins with this sequence have generally been considered to be poor substrates for soluble CDPKs tested to date (Roberts, 1989; Polya *et al.*, 1989; Putnam-Evans *et al.*, 1990). The apparent contradiction of this proposed recognition sequence with data available for other CDPKs may be due to a slightly different specificity for the tonoplast enzyme. Also, the co-localization of a protein kinase and substrate to a particulate structure such as a membrane often can result in phosphorylation events that normally would not occur in

solution (Hubbard & Cohen, 1993). The phosphorylation site on α-TIP also contains a basic residue two residues carboxyl-terminal to ser[7], which may be important based on the S-X-R/K motif proposed by Polya *et al.* (1989). However, it is still unclear which residues are important for CDPK's recognition of this substrate.

Nodulin 26 contains five R/K-X-X-S motifs (Fig. 5). Thus, nodulin 26 is an attractive model to begin to study the phosphorylation site recognition requirements for an endogenous CDPK substrate. The results presented here show that the site in nodulin 26 and CK-15 that is phosphorylated by the SM-associated CDPK is a single serine, ser[262] in nodulin 26 and the corresponding ser[6] in CK-15 (Weaver & Roberts, 1992). This site is found in the sequence T-K-S-A-S^{PO4}-F-L-K-G-R-A-A-S-K. It is worthy of note that ser[262] is unique since it is the only one of the five sites in nodulin 26 containing the R/K-X-X-S motif that does not have a charged amino acid residue next to the potential phosphorylation site. The importance of this observation has not been investigated experimentally, and the actual determinants within the CK-15 region of nodulin 26 that are required for recognition by CDPK remain unclear. However, these results do suggest that other residues surrounding the phosphorylation site play a role in recognition and that the R/K-X-X-S motif is not the only determining factor in CDPK recognition of the phosphorylation site.

Besides primary amino acid sequence requirements, secondary or tertiary structural constraints of nodulin 26 may play a role in the recognition of only ser^{262} by CDPK. Studies of other protein kinase recognition sequences have revealed that not all of the consensus recognition motifs found in proteins are phosphorylated (Kemp & Pearson, 1990). This observation has led to the conclusion that higher orders of structure are overriding considerations in determining whether or not a given site may be recognized and phosphorylated (Kemp & Pearson, 1990). The results from the determination of the nodulin 26 site indicate that a more thorough investigation of the determinants of CDPK phosphorylation site recognition in this substrate need to be performed. Such an approach might include the systematic substitution of residues surrounding the CK-15 site. A similar approach has been used for other protein kinases (reviewed in Pearson & Kemp, 1991).

Nodulin 26 functional characterization

The results presented in this report establish the localization of nodulin 26 in the SM and suggest that nodulin 26 may be a target of Ca^{2+}-dependent regulation by CDPK. These findings raise the question of how nodulin 26 may be involved in SM function and how Ca^{2+} might control this function. Previous reports have provided a body of information that supports a role for the SM in metabolic flux between the host plant cytoplasm and the endosymbiotic bacteroid (Day *et al.*, 1990; Day & Udvardi, 1993). Among the

SM-transport activities associated with soybean symbiosomes are: a malate transport activity (Udvardi *et al.*, 1988), a H^+-pumping ATPase activity (O'Niell *et al.*, 1983; Blumwald *et al.*, 1985; Udvardi and Day, 1989; 1991), and an anion transport activity (Udvardi *et al.*, 1989; 1991). The identity of the proteins which mediate these functions, as well as information on regulation of their activities is lacking.

The finding that nodulin 26 is structurally homologous to channel proteins has led to speculation that it is a SM channel (Sandal & Marcker, 1990). Prior to the present study, however, no evidence existed to support such a presumption. By using the knowledge that nodulin 26 was the only detectable phosphoprotein in the SM, and that it was phosphorylated by a SM-associated CDPK, we attempted to investigate the effects of phosphorylation of nodulin 26 on SM malate transport by using intact symbiosomes. The results of this investigation show a correlation between the phosphorylation state of nodulin 26 and the rate of malate uptake in isolated symbiosomes. In particular, conditions that favor phosphorylation of nodulin 26 also favor enhanced malate uptake rates. Although suggestive, these data did not directly establish that nodulin 26 was responsible for the malate uptake activity of the SM.

To obtain direct biochemical proof that nodulin 26 can function as a transporter, the protein was purified and analyzed by single channel measurements in planar lipid bilayers. By this approach it has been demonstrated that

nodulin 26 forms a channel that transports ions across a lipid bilayer. The characterization of the properties of nodulin 26 channels show that it has a high unitary conductance, up to 3.1 nS, and that it shows a low selectivity for Cl^- over K^+. Furthermore, we have observed that the nodulin 26 channel is a voltage-sensitive channel that has a tendency to occupy lower conductance states at high applied voltages. The data gained by examining the properties of purified nodulin 26 in planar lipid bilayers has provided the first direct functional analysis of the nodulin 26 protein.

In a preliminary experiment, it appeared that the nodulin 26 channel will conduct malate, which is consistent with its proposed role as a SM metabolite transporter. However, in addition to a potential role in malate transport, the low specificity of the nodulin 26 channel for ions suggest that it can participate in other SM transport activities. For example, the low specificity of the nodulin 26 channel suggest that it could be responsible for the general anion transport properties attributed to the SM (Udvardi *et al.*, 1989; 1991) as well as other transport properties that may be required for the maintenance of the bacteroid. Thus, it is conceivable that the nodulin 26 channel may act in a more general role for the transport of metabolites, ions, and co-factors between the plant cytosol and the bacteroid.

A more thorough investigation of the conductance properties of nodulin 26 needs to be performed in order to

better define the specificity of the nodulin 26 ion channel. A comparison of the ion selectivity properties of the nodulin 26 channel with the general anion transporter of the SM would be useful in this regard. In addition, assays of nodulin 26's ability to transport uncharged molecules are necessary to define its transport selectivity. Channel measurements also need to be performed using purified SM since it is possible that an accessory protein may act as an effector of the nodulin 26 channel activity. Such a protein would not have been present in channel experiments using purified nodulin 26. In support of this proposition, exogenous accessory proteins affect the MIP channel (Ehring *et al.*, 1990).

The observation that the nodulin 26 channel exhibits voltage-dependent channel closure suggests that this may be a mechanism for its regulation. As mentioned previously, an SM-associated H^+-pumping ATPase is able to establish an electrochemical gradient across the SM and this gradient has been implicated in the control of SM malate and anion uptake (Ou Yang *et al.*, 1990; Udvardi *et al.*, 1989; 1991). Although the magnitude of the electrochemical gradient across the SM has not been quantitated, the resting state electrical potentials across other plant membrane systems such as the plasma membrane and the tonoplast are known to exceed 100 mV (Miller *et al.*, 1990). If a potential of this magnitude exists across the SM, the nodulin 26 ion channel could occupy its lower conductance states or it could be closed based on

its properties measured in planar lipid bilayers. Thus, in addition to providing a driving force for directional ion transport, trans-SM potentials could also serve to control nodulin 26 transport by stimulating channel closure at higher potentials.

Nodulin 26 and the MIP family

Over the past few years, the number of proteins in the MIP family has grown quickly, with eighteen-members identified from complete or partial sequences (Reizer *et al.*, 1993). These proteins are found in a variety of organisms that span the phylogenetic spectrum, including members from bacteria, animals, plants, and yeast. Among the identified members of the family only MIP, *GlpF*, CHIP, γ-TIP, and nodulin 26 have been functionally evaluated. Similar to nodulin 26, MIP is an high-conductance, low-specificity, voltage-sensitive ion channel (Ehring *et al.*, 1990). *GlpF* transports small straight chain polyols (Heller *et al.*, 1980; Maurel *et al.*, 1993). CHIP is a water-channel in erythrocytes and renal tubules (Preston *et al.*, 1992; Zeidel *et al.*, 1992; van Hoek & Verkman, 1992), and γ-TIP is a water channel in plants (Maurel *et al.*, 1993).

Comparisons of the deduced amino acid sequences (Fig. 3) of these proteins has led to some interesting initial observations. A number of highly conserved amino acid residues have been identified among the MIP family members (Reizer *et al.*, 1993). For instance, there is an invariant D-

P-N sequence in the loops between the second and third and between the fifth and sixth putative transmembrane domains (Fig. 4). Also, there is a highly conserved E-X-X-S/T sequence in the first and fourth proposed transmembrane helices (Fig 4). Because of their location within putative transmembrane regions, it has been suggested that the hydrophilic E and S/T residues may be involved in lining the channel pore (Reizer *et al.*, 1993). There is also a conserved G residue found in the third and sixth proposed transmembrane domains. In other more well characterized channels the existence of hydrophilic and small side chain residues have been shown to be important in formation of the channel pore and in channel function (Unwin, 1993).

In terms of their phylogenetic relationships, it appears that the plant proteins such as the members of the TIP subfamily and nodulin 26, group together based on deduced amino acid sequence homology (Reizer *et al.*, 1993). Similarly, the members identified from the animal kingdom such as MIP and CHIP appear to be more closely related to one another than to bacterial and plant homologs (Reizer *et al.*, 1993). The other members of the family form distinct branches with large evolutionary differences separating each of them (Reizer *et al.*, 1993).

As mentioned previously, based on the phylogenetic tree, MIP and CHIP are most closely related. However, single channel measurements of MIP in planar lipid bilayers show

that, similar to nodulin 26, it is a high conductance, low specificity, voltage-sensitive ion channel, whereas CHIP appears to be a specific water channel. Therefore, although MIP and CHIP are the most closely related MIP family members in terms of primary amino acid sequence, they appear to possess very different biochemical activities.

A similar observation can be made with γ-TIP and nodulin 26. It has been shown that expression of γ-TIP cRNA in *Xenopus* oocytes resulted in the expression of water channel activity with properties very similar to CHIP (Maurel *et al.*, 1992). These similarities include enhanced water transport rates, high specificity for water transport, and inhibition by $HgCl_2$. However, as shown in this report, purified nodulin 26 is a high conductance ion channel with properties very similar to MIP (Ehring *et al.*, 1990; Weaver *et al.*, submitted). Despite this functional similarity, nodulin 26 shows higher sequence homology to γ-TIP than to MIP (Reizer *et al.*, 1993). *GlpF* can not transport water, when expressed in *Xenopus* oocytes, but it can transport glycerol (Maurel *et al.*, 1993). This is consistent with its reported function assayed *in situ* in *E. coli* (Heller *et al.*, 1980; Linn, 1986). Thus, there are at least three distinct activities associated with MIP family members: ion channels, water channels, and the transport of small uncharged molecules.

Considered together, these data suggest that evolutionary distance and the specific biochemical functional relationships

of the members of this family may differ considerably. Thus, the specific structural features that determine these diverse functions of the members of the MIP family remain uncertain. However, as the number of biochemical activities associated with MIP family members grows, it will be interesting to compare the amino acid sequence similarities of the proteins with similar functional properties. This approach may help identify amino acids residues that led to the divergent functions of the members of this family. This will provide a data base of information to allow a more rational approach in designing experiments to elucidate how the diverse functions of these structurally-related proteins are determined.

Regulation of nodulin 26 ion channels by phosphorylation

The regulation of ion channel activity by phosphorylation is a common occurrence. Phosphorylation regulates the activity of numerous channel proteins including acetylcholine receptor ion channels (Huganir *et al.*, 1986), GABA receptor ion channels (Huganir & Greengard, 1990), glutamate receptor ion channels (Raymond *et al.*, 1993), glycine receptor ion channels (Song & Huang, 1990), neuronal K^+ channels (Adams & Levitan, 1982; Benson & Levitan, 1983), and cardiac Ca^{2+} channels (Osterrieder *et al.*, 1982; Brum *et al.*, 1983). The effects of phosphorylation on these channels include effects on desensitization rates (Huganir *et al.*, 1986), increases in the frequency and duration of channel opening (Song & Huang,

1990), and the stimulation of channel closure (Benson & Levitan, 1983). Phosphorylation has also been observed to affect the subunit assembly and aggregation of some ligand gated ion channels (Wallace *et al.*, 1991). Therefore, the potential regulatory effects of phosphorylation on ion channels are quite varied.

Far less investigation has been performed with regard to the regulatory role of phosphorylation on plant ionic conductances. This is due, in large degree, to the fact that relatively little is known about plant ion channel activity. Of the plant ion channel activities thus far described, the only potential effects of phosphorylation on ion channel activity come from ion channels observed in guard cell protoplasts. The PM K^+ channel has been observed to display Ca^{2+}-dependent inactivation (Schroeder & Hagiwara, 1989). Recently this inactivation has been correlated with a dephosphorylation event that is proposed to be mediated by a plant isoform of the Ca^{2+}/calmodulin mediated phosphatase calcineurin (Luan *et al.*, 1993). This correlation is based primarily on the pharmacological effects of the calcineurin inhibitor cyclosporin/cyclophillin complex (Luan *et al.*, 1993).

Of the proteins in the MIP family, only MIP and nodulin 26 have been studied with respect to the influence of phosphorylation on ion channel activity. It has been known for some time that MIP is phosphorylated by protein kinase C

(Lampe *et al.*, 1986) and the cyclic AMP-dependent protein kinase (Johnson *et al.*, 1986). The phosphorylation by cyclic AMP-dependent protein kinase has been documented to occur *in vivo* (Lampe & Johnson, 1990). The sites of phosphorylation of MIP by cyclic AMP-dependent protein kinase has been established as ser^{243} (Lampe & Johnson, 1990). This residue is in the hydrophilic carboxyl-terminal portion of the molecule in a similar location as the phosphorylation site of nodulin 26 (Weaver and Roberts, 1992).

Recently, the functional effects of phosphorylation of MIP by cyclic AMP-dependent protein kinase have been evaluated (Ehring *et al.*, 1991). By using ion-exchange chromatography, samples enriched in phosphorylated and unphosphorylated MIP were obtained and assayed in planar lipid bilayers (Ehring *et al.*, 1991). Unphosphorylated MIP displayed a channel activity that was virtually insensitive to voltage. In contrast, phosphorylated MIP channels displayed the voltage-sensitive profile previously reported for the MIP channel (Ehring *et al.*, 1990). Furthermore, the unphosphorylated, voltage-insensitive channel could be converted to the voltage-sensitive form *in situ* by the addition of the catalytic subunit of cyclic AMP-dependent protein kinase and ATP to the bilayer chamber (Ehring *et al.*, 1991). The effects of phosphorylation with regard to sensitivity of MIP to applied voltage appear to result from an increase in the closed channel life-time as well as an increase in the rate of

closure of the channel in response to applied voltage (Ehring *et al.*, 1991). However, phosphorylation does not appear to significantly affect the single channel conductance (Ehring *et al.*, 1991). These data support the suggestion that the carboxyl-terminus of MIP is important in mediating the voltage sensitivity of the channel. In this regard, the carboxyl-terminal region might serve as a channel "gate", which might control channel conductance by interacting with the channel pore. This is consistent with the previous observation that limited proteolysis of the carboxyl terminus of MIP led to decreased voltage sensitivity (Ehring *et al.*, 1990).

As reported in the present study, nodulin 26 is also the target of phosphorylation both *in vitro* and *in vivo*. Preliminary evidence suggests that purified nodulin 26 assayed in planar lipid bilayers occupies its higher conductance states in the presence of high applied voltage after the addition of alkaline phosphatase to the bilayer chamber. Thus, phosphorylation of nodulin 26 by CDPK may render it sensitive to voltage-induced channel closure, which is similar to the effect of phosphorylation of MIP by cyclic AMP-dependent protein kinase. This observation also suggests that isolated nodulin 26 channels measured in this study may have been already phosphorylated since they displayed voltage-sensitive channel closure.

One possible role of nodulin 26 phosphorylation could be the modulation of nodulin 26 transport activity *in vivo* by

conferring sensitivity to closure by transmembrane potentials generated by the SM-H^+-pumping ATPase. However, it is difficult to reconcile this apparent inhibitory effect of nodulin 26 phosphorylation on its channel activity with the apparent stimulatory effects of phosphorylation on malate uptake in intact symbiosomes (Ou Yang *et al.*, 1991). One problem with this model is the uncertainty of whether malate is transported by a specific malate carrier (Udvardi *et al.*, 1988; Ou Yang *et al.*, 1990) and/or by a general anion transport mechanism (Udvardi *et al.*, 1989; 1991). If these two protein activities are distinct, then one could propose a possible scenario to resolve the apparent contradiction in the positive effects of phosphorylation on malate transport in intact symbiosomes and the apparent negative effects of phosphorylation on nodulin 26 channel activity. As discussed above, based on its ion transport specificity, it is possible that nodulin 26 is related to the anion transport. If nodulin 26 is responsible for the general SM anion transport activity, a phosphorylated, and therefore potentially closed nodulin 26 channel might allow the preservation of the trans-SM potential for malate transport across the SM via the dicarboxylate carrier. Experiments designed to test this possibility need to be done. In particular, the specific SM proteins responsible for these activities and their relationship to nodulin 26 need to be assessed.

As a final note, it has been observed that another MIP

analog, the seed protein α-TIP, is phosphorylated by a tonoplast-associated CDPK (Johnson & Chrispeels, 1992). Furthermore, an increase in the apparent phosphorylation level of α-TIP is related to the age of the cotyledon after germination (Johnson & Chrispeels, 1992). Since no biochemical activity has been described for α-TIP, however, it is not clear what effects this phosphorylation event may mediate. However, phosphorylation of MIP analogs in plants by CDPK may play an important as yet undefined role in transport.

Summary and prospects

In summary, the studies described in this thesis represent the first detection and localization of nodulin 26 protein in soybean root nodules, the establishment of nodulin 26 as a target of phosphorylation for a soybean nodule CDPK, the determination of the site of phosphorylation of an endogenous substrate for the CDPK family of protein kinases, and the demonstration that nodulin 26 forms a high conductance, weakly anion-selective, voltage-sensitive channel. These data suggest a potential role for this nodule-specific SM protein in SM transport functions and, a potential role for Ca^{2+}-mediated signal transduction in the control of nodule metabolism and host/symbiont communication. Clearly, a more detailed investigation needs to be performed with regard to the transport properties of nodulin 26 and the effects of phosphorylation by CDPK. In addition, an investigation of the environmental stimuli that influence CDPK

activity and thus the phosphorylation state of nodulin 26 needs to be undertaken. Hopefully, the data gained from this investigation will provide further insight into the physiological role of nodulin 26 in the maintenance and control of the soybean/rhizobial nitrogen-fixing symbiosis.

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VITA

Charles David Weaver was born in Knoxville, Tennessee on May 29, 1967. He graduated from Clinton Senior High School in Clinton, Tennessee in June, 1985. He entered the University of Tennessee, Knoxville in August, 1985 and graduated with a Bachelor of Science degree in Cell Biology with a minor in Botany, in May, 1989. Beginning with the summer of his Sophomore year at the University, he was employed as a technician in the departments of Microbiology and Biochemistry, where he developed an interest in calcium signal transduction.

He began his graduate study in August, 1989 at the University of Tennessee, Knoxville in the Department of Biochemistry and received a Ph.D. in May, 1994. He was employed at the University of Tennessee as a GTA (graduate teaching assistant) from August, 1989 to December, 1993 and as a post-doctoral associate from January, 1993. During the period of his graduate work he presented his research data through oral and poster presentations at several scientific meetings. He also presented his data in departmental journal meetings and departmental seminar. Finally, as a result of his graduate research, he published several papers (see below for details).

Published Papers and Abstracts

(1) D.M. Roberts, S.-H. Oh, L. Besl, **C.D. Weaver**, & Gary Stacey. (1990) Attenuation of calmodulin-dependent NAD kinase by posttranslational calmodulin methylation. *in* Current Topics in Plant Biochemistry and Physiology, **9**, 67-84.

(2) D.M. Roberts & **C.D. Weaver**. (1990) How plants respond to stimuli. *New Biologist* **2**, 678-683.

(3) **C.D. Weaver**, B. Crombie, G. Stacey, & D.M. Roberts. (1991) Calcium-dependent phosphorylation of symbiosome membrane proteins from nitrogen-fixing root nodules: evidence for the phosphorylation of nodulin 26. *Plant Physiol.* **95**, 222-227.

(4) L.-J. Ou Yang, J. Whelan, **C.D. Weaver**, D.M. Roberts, & D.A. Day. (1991). Protein phosphorylation stimulates the rate of malate uptake across the peribacteroid membrane of soybean nodules. *FEBS Lett.* **293**, 188-190.

(5) D.M. Roberts, **C.D. Weaver**, & S.-H. Oh. (1992) Intracellular receptor proteins for calcium signals in plants. *in* Plant Biotechnology and Development (Gresshoff, P.M., Ed.) pp 129-138, CRC Press, Boca Raton, FL.

(6) **C.D. Weaver**, & D.M. Roberts. (1992) Determination of the site of phosphorylation of nodulin 26 by the calcium-dependent protein kinase from soybean nodules. *Biochemistry*. **31**, 8954-8959.

(7) R. Nichols, **C.D. Weaver**, E. Eisenstein, T.-H. Huang, F.-Y. Huang, & E.E. Howell. (1993) Titration of histidine 62 in R67 dihydrofolate reductase is linked to a tetramer <=> 2 dimer equilibrium. *Biochemistry*. **32**, 1695-1706.

(8) **C.D. Weaver**, N.H. Shomer, C.F. Louis, & D.M. Roberts. Nodulin 26, a nodule-specific symbiosome membrane protein from soybean, is an ion channel. Submitted for review.

Work in Preparation

(1) Y. Zhang, **C.D. Weaver**, & D.M. Roberts. Effect of the expression of nodulin 26 on the growth and development of tobacco plants. In preparation.

Presentations at Scientific Meetings

(1) D.M. Roberts, **C.D. Weaver**, L.E. Roth, & G. Stacey. (1988) Potential targets of calmodulin in wild-type and mutant soybean root nodules. 4th International Symposium on Molecular Genetics of Plant Microbe Interactions.

(2) **C.D. Weaver**, G. Stacey, & D.M. Roberts. (1990) Phosphorylation of nodulin 26 by a calcium-dependent protein kinase. *Plant Physiol. Suppl.* **93**, 18.

(3) **C.D. Weaver**, G. Stacey, & D.M. Roberts. (1990) Phosphorylation of nodulin 26 by a calcium-dependent protein kinase. *in* Proceedings of the 8th International Congress on Nitrogen Fixation. **8**, 774.

(4) R. Nichols, **D. Weaver**, L. Reece, & E. Howell. (1990) Characterization of a novel plasmid encoded (R67) dihydrofolate reductase (DHFR). 4th Symposium of the Protein Society.

(5) R. Nichols, **D. Weaver**, L. Reece, & E. Howell. (1991) Characterizations of subunit interactions of a type II DHFR. *FASEB J.* **5**, A186.

(6) **C.D. Weaver**, & D.M. Roberts. (1991) Phosphorylation of nodulin 26 by a calcium-dependent protein kinase. *FASEB J.*, **5**, A427.

(7) **C.D. Weaver**, L.-J. Ouyang, D.A. Day, & D.M. Roberts. (1992) Structural and functional characterization of nodulin 26 phosphorylation by the calcium-dependent protein kinase. *Mol. Biol. Cell Suppl.* **3**, 716.

(8) D.M. Roberts, **C.D. Weaver**, L.-J. Ouyang, & D.A. Day. (1992) Structural and functional characterization of nodulin 26 phosphorylation by the calcium-dependent protein kinase. 9th International Congress on Nitrogen Fixation, 236.

(9) **C.D. Weaver**, N.H. Shomer, C.F. Louis, & D.M. Roberts. (1993) Ion channel activity of nodulin 26 isolated from nitrogen-fixing soybean root nodules. *Mol. Biol. Cell Suppl.* **4**, 2468.

(10) Y. Zhang, **C.D. Weaver**, & D.M. Roberts. (1993) Effect of the expression of nodulin 26 on the growth and development of tobacco plants. *Mol. Biol. Cell Suppl.* **4**, 2469.

(11) N.H. Shomer, **C.D. Weaver**, D.M. Roberts, & C.F. Louis. (1994) Single channel properties of the nodulin 26 protein from soybean symbiosome membranes. to be presented at the Biophysical Society meeting, 1994.

Seminars

(1) Invited speaker, 8th International Congress on Nitrogen Fixation. May, 1990.

(2) Invited speaker, 3rd Gatlinburg Symposium, Plant Response to the Environment. June, 1992.

(3) Seminar, Department of Biochemistry, University of Tennessee, Knoxville, December, 1993.