Interaction of Cytosolic Glutamine Synthetase of Soybean Root Nodules with the C-terminal Domain of the Symbiosome Membrane Nodulin 26 Aquaglyceroporin

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Nodulin 26 (nod26) is a major intrinsic protein that constitutes the major protein component on the symbiosome membrane (SM) of N₂-fixing soybean nodules. Functionally, nod26 forms a low energy transport pathway for water, osmolytes, and NH₃ across the SM. Besides their transport functions, emerging evidence suggests that high concentrations of major intrinsic proteins on membranes provide interaction and docking targets for various cytosolic proteins. Here it is shown that the C-terminal domain peptide of nod26 interacts with a 40-kDa protein from soybean nodule extracts, which was identified as soybean cytosolic glutamine synthetase GS₁β₁ by mass spectrometry. Fluorescence spectroscopy assays show that recombinant soybean GS₁β₁ binds the nod26 C-terminal domain with a 1:1 stoichiometry \((K_d = 266 \text{ nM})\). GS₁β₁ also binds to isolated SMs, and this binding can be blocked by preincubation with the C-terminal peptide of nod26. In vivo experiments using either a split ubiquitin yeast two-hybrid system or bimolecular fluorescence complementation show that the four cytosolic GS isoforms expressed in soybean nodules interact with full-length nod26. The binding of GS, the principal ammonia assimilatory enzyme, to the conserved C-terminal domain of nod26, a transporter of NH₃, is proposed to promote efficient assimilation of fixed nitrogen, as well as prevent potential ammonia toxicity, by localizing the enzyme to the cytosolic side of the symbiosome membrane.

The formation of legume-rhizobia N₂-fixing symbioses in root nodules leads to the development of a novel organelle known as the “symbiosome,” which houses the endosymbiotic rhizobia bacteroids. In mature nodules, the host infected cells are occupied by thousands of symbiosomes, which constitute the major organelle within this specialized cell type. The symbiosome is delimited by the symbiosome membrane (SM),

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1 The abbreviations used are: SM, symbiosome membrane; nod26, nodulin 26; GS, glutamine synthetase; MIP, major intrinsic protein; ORF, open reading frame; Ni-NTA, nickel-nitrilotriacetic acid; BiFC, bimolecular fluorescence complementation; NBD, nitrobenzoxadiazole; YFP, yellow fluorescent protein; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
nod26 serves as a site for the association of cytosolic glutamine synthetase (GS) with the SM, suggesting novel roles for nod26 in ammonia transport and assimilation.

**EXPERIMENTAL PROCEDURES**

**Purification of Nodule Glutamine Synthetase**—Soybean ( Glycine max cv Bragg) nodulated with Bradyrhizobium japonicum USDA110 were grown as described in Ref. 5. Symbiosome membranes were isolated as described in Refs. 5 and 6. For the preparation of soybean nodule GS, nodules were homogenized in 100 mM Tris-HCl (pH 8.4), 10 mM MgOAc, 10% v/v glycerol, 0.05% v/v Triton X-100 (3 ml/g of nodules) on ice. The extract was centrifuged at 35,000 × g at 4 °C for 30 min, and the supernatant proteins were precipitated with an equal volume of chilled acetone. The precipitate was collected and resuspended in 10 mM Tris-HCl (pH 7.5), 10 mM MgOAc, 10% v/v glycerol (Sephacryl Buffer) and subjected to differential (NH4)2SO4 precipitation. The pellet obtained from the 30—60% saturation cut was resuspended in Sephacryl Buffer and chromatographed at 4 °C on Sephacryl S300 (50 × 2-cm column). Fractions with maximal GS activity were pooled and stored at −80 °C.

**Molecular Cloning of GS, Isoforms from Nitrogen-fixing Soybean Root Nodules**—Total RNA was extracted from 35-day-old soybean nodules by using the PLANT RNA extraction reagent (Invitrogen). Total cDNA was prepared using the SuperScript III reverse transcription kit (Invitrogen). PCR amplification of cDNAs encoding GS1, isoforms GS1β1, GS1β2, GS1γ1, and GS1γ2 (23) was done from soybean nodule cDNA using primers against the 5′- and 3′-untranslated regions of each isoform (supplemental Table 1) based on sequences available from the genomic data base Phytozome. PCR products were cloned into pCR2.1-TOPO (Invitrogen) and transformed into Escherichia coli DH5α. Isoform identity (supplemental Fig. 1) was verified by automated DNA sequence analysis.

**Expression and Purification of Recombinant GS1β1**—A cDNA containing the full-length open reading frame (ORF) of soybean cytosolic GS1β1 was cloned into pDest17 in-frame with an amino-terminal His tag linker by using the Gateway Cloning Technology (Invitrogen). Recombinant GS1β1 expression in E. coli BL21 was induced in mid-log cultures with 1 mM isopropyl β-D-1-thiogalactopyranoside. Cultures were grown with shaking for 16 h at room temperature. Cells were collected and resuspended in 20 mM Tris-HCl (pH 7.9), 300 mM NaCl, 25 mM imidazole, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 0.1% (w/v) Triton X-100 and lysed in a French press pressure cell (SLM-AMINCO Spectrographic Instruments). The lysate was centrifuged at 150,000 × g for 30 min at 4 °C, and the supernatant fraction was chromatographed on Ni2+-nitrilotriacetic acid (Ni-NTA)-agarose pre-equilibrated in NTA Buffer (20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 5% (v/v) glycerol) containing 20 mM imidazole. The column was washed with NTA Buffer containing 60 mM imidazole and eluted with NTA Buffer containing 500 mM imidazole. Fractions containing GS activity were pooled, dialyzed against 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl2, and stored at −80 °C.

**Affinity Chromatography on Peptide Resins**—A synthetic peptide (CK-25), which contains the entire C-terminal region of soybean nod26 (see Fig. 1) with an additional N-terminal cysteine, was obtained from GenScript (Piscataway, NJ). The CI-14 peptide, corresponding to the C terminus of Lotus japonicus nod26, was prepared as described in Ref. 9. For the purpose of this study, identical results were obtained regardless of which nod26 peptide was used. Immobilized peptide resins were prepared by covalent coupling to ω-aminohexyl-agarose by the protocol in Ref. 9. Soybean nodule extracts were prepared as described above, and 5 ml (1 mg/ml protein) were applied to resin (0.2 ml) equilibrated in 50 mM Tris-HCl, 150 mM NaCl, pH 7.5 (Binding Buffer). After extensive washing, the resin was eluted with 50 mM Tris-HCl, pH 7.5, 6 μl urea, and the eluent was analyzed by SDS-PAGE on 12.5% (w/v) polyacrylamide gels using the buffer system of Laemmli (24).

For resin association assays, 50 μl of CK-25 peptide resin or underivatized ω-aminohexyl-agarose (negative control) were incubated with 50 units (1 unit = 1 nmol of γ-hydroxyglutamate/min at 37 °C) of purified soybean GS in Binding Buffer for 30 min at 25 °C with intermittent mixing. The resin was separated from the soluble fraction by centrifugation and washed with 10 μl of Binding Buffer. The fraction of the GS activity bound to the resin or present in the unadsorbed supernatant fractions was determined.

**Two-dimensional Electrophoresis**—For two-dimensional electrophoresis, protein samples (5 μg) were dissolved in a final volume of 150 μl of 8 M urea, 2% (v/w) octylglucoside, 4% (w/v) CHAPS, 1% (w/v) dithiothreitol, 0.16% (v/v) BioLytes 5–7, 0.04% (v/v) BioLytes 3–10. Separation of samples in the first dimension was done by isoelectric focusing on 7-cm ReadyStrip IPG strips (immobilized linear pH 5–8 gradient from Bio-Rad) in a PROTEAN isoelectric focusing cell apparatus (Bio-Rad) at 20 °C. Isoelectric focusing was done using the following five steps: 100 V for 200 V-h, 500 V for 500 V-h, 1000 V for 1000 V-h, 1000–8000 V for 1 h, and maintained at 8000 V for 8000 V-h. The IPG strips were equilibrated twice for 15 min in 5 ml of 50 mM Tris-HCl, pH 6.8, 6 M urea, 20% (v/v) glycerol, and 2% (w/v) SDS. Two percent (v/v) dithiothreitol was added during the first equilibration step followed by the addition of 2.5% (w/v) iodoacetamide during the second equilibration step. The IPG strips were placed on top of the 8.5% (w/v) polyacrylamide gels for second-dimension separation by SDS-PAGE.

**Mass Spectrometry**—Proteins resolved by SDS-PAGE were identified by in-gel tryptic digestion followed by peptide mass fingerprinting as described in Ref. 25. Digested peptides were extracted in 0.1% (v/v) trifluoroacetic acid and 60% (v/v) acetonitrile, and the extracted samples were desalted and concentrated with a 10-μl ZipTipC18 (Millipore, Bedford, MA) following the instructions provided by the manufacturer. The final sample was mixed with α-cyano-4-hydroxy-cinnamic acid dissolved in 60% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid. Peptide mass spectra were acquired on a Bruker microflex time-of-flight mass spectrometer (Bruker Daltonics) with a nitrogen laser operating at 337 nm on a positive ion mode. The masses of tryptic peptides were analyzed by searching the National Center for Biotechnology Information (NCBI) nonredundant protein data base with ProFound tool from The Rockefeller University Laboratory of Mass Spectrometry and Gaseous Ion Chemistry web site. Peptide masses were assumed to be monoisotopic, and methionine residues were assumed to be...
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partially oxidized. A maximum of two missed tryptic cleavages were allowed. Tandem mass spectrometry sequence analysis of in-gel tryptic digests was done at the University of Georgia Proteomics Facility.

**Peptide and SM Binding Assays**—CK-25 peptide (0.42 μmol) was dissolved in 75 μl of 50 mM Tris-HCl, pH 7.5, and labeled at 4 °C overnight with a 10-fold molar excess of N,N,N’-dimethyl-N’-(iodoacetyl)-N”-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine (Molecular Probes). The nitrobenzoxadiazole (NBD)-labeled peptide was isolated from excess enediamine (Molecular Probes). The nitrobenzoxadiazole (NBD)-labeled peptide was isolated from excess N,N’-dimethyl-N’-(iodoacetyl)-N”-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine reagent by chromatography on a Sephadex G-25 in 50 mM Tris-HCl (pH 7.5). The labeled peptide concentration was calculated by absorbance (NBD ε = 26,000 M⁻¹cm⁻¹), and residual unlabeled peptide was quantitated by using Ellman’s reagent. The increase in fluorescence intensity as a function of added GS was fit to the following binding expression

\[
\Delta F = \frac{\Delta F_{\text{max}} [\text{GS}]}{K_d + [\text{GS}]} \quad (\text{Eq. 1})
\]

where \(\Delta F\) is the change in fluorescence intensity, \(\Delta F_{\text{max}}\) is the maximal change in fluorescence intensity at saturation, [GS] is the concentration of GS, and \(K_d\) is the dissociation constant.

For SM binding experiments, 100 μg of SM were incubated with 50 units of purified soybean nodule GS in Binding Buffer with 5 mM MgCl₂ for 1 h at 4 °C. Membranes were collected by centrifugation at 200,000 × g for 5 min prior to fluorescence measurements (excitation λ = 480 nm, emission λ = 545 nm). The increase in fluorescence intensity as a function of added GS was fit to the following binding expression

\[
\Delta F = \frac{\Delta F_{\text{max}} [\text{GS}]}{K_d + [\text{GS}]} \quad (\text{Eq. 1})
\]

where \(\Delta F\) is the change in fluorescence intensity, \(\Delta F_{\text{max}}\) is the maximal change in fluorescence intensity at saturation, [GS] is the concentration of GS, and \(K_d\) is the dissociation constant.

For SM binding experiments, 100 μg of SM were incubated with 50 units of purified soybean nodule GS in Binding Buffer with 5 mM MgCl₂ for 1 h at 4 °C. Membranes were collected by centrifugation at 200,000 × g for 30 min and washed with 2 ml of Binding Buffer with 5 mM MgCl₂. The centrifugation-washing cycle was repeated three additional times, and the membranes were resuspended in 100 μl of Binding Buffer and assayed for GS activity.

**Yeast Split Ubiquitin Analyses**—Split ubiquitin yeast two-hybrid experiments were performed as described in Ref. 27. THY.AP4 and THY.AP5 *Saccharomyces cerevisiae* strains as well as the pMetYCgate, pNXgate32, pNubWT, KAT1/pMetYCgate, and KAT1/pNXgate32 vectors were obtained from the Arabidopsis Biological Resource Center (Ohio State University). The nod26 bait construct containing the C terminus of ubiquitin (Cub) was generated by cloning the soybean nod26 ORF into the pMetYCgate vector in the THY.AP4 strain. The nod26 construct containing the N-terminal ubiquitin fragment (Nub) was generated by cloning the respective ORFs in the pNXgate32 vector in the THY.AP5 strain. The primers used in the preparation of all the constructs are listed in supplemental Table 1. Interaction assays were done by mating various bait and prey pairs (Basic protocol 2 in Ref. 28). Diploids were selected by replica plating on synthetic medium plates lacking tryptophan, leucine, and uracil. For β-galactosidase selection, diploids were grown on selective medium for 2 days at 30 °C and overlaid with 60 mM sodium phosphate, pH 7.2, 0.2% sodium dodecyl sulfate, 2 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 0.5% (w/v) agarose. Plates were stored at 37 °C overnight to allow color development. For HIS3 selection, diploid cells were replica-plated and grown on a synthetic dextrose medium lacking histidine, leucine, tryptophan, and uracil for 2 days at 30 °C. Growth was scored by comparison of test yeast colonies to control yeast colonies containing empty pMetYCgate and pNXgate vectors.

**Bimolecular Fluorescence Complementation (BiFC) Assay**—Constructs containing soybean nodule GS, β1 or nod26 coding sequences as translational fusions with either the N-terminal 154 residue fragment (YFP-N) or the C-terminal 84 residue fragment (YFP-C) of the yellow fluorescent protein were prepared in pd35S-YFP-N or pd35S-YFP-C vectors (a kind gift from Dr. Andreas Nebenführ, the University of Tennessee, Knoxville, TN). Prior to cloning into BiFC plasmids, the BamHI restriction site in GS, β1 was removed by PCR-based site-directed mutagenesis (29). The N-terminal fusions of GS, β1 and nod26 (YFP-C, GS, β1 and YFP-N, nod26) were prepared by cloning ORFs of GS, β1 and nod26 into BamHI-NotI-digested pd35S-YFP-C and pd35S-YFP-N, respectively. The C-terminal fusion of nod26 (nod26-YN) was prepared by cloning the nod26 ORF into XbaI-BamHI-digested pd35S-YFP-N. In all constructs, a linker region of 10 residues (GGHHHHHHGG) was introduced between the YFP fragment and the ORF of interest. All primers used for molecular cloning are listed in supplemental Table 1.

Transient expression of fusion proteins and visualization of BiFC interactions were done by tungsten particle bombardment of onion bulb epidermal cells as described previously (30, 31). Bombarded onion cells were incubated for 24 h at 28 °C prior to microscopic examination with an Axiosvert 200 M microscope (Zeiss) equipped with filters for YFP fluorescence (Chroma, filter set 52017). Images were captured with a digital camera (Hamamatsu Orca-ER) controlled by the Openlab software (Improvision).

**Other Analytical Methods**—GS activity was assayed by the hydroxylamine colorimetric method (32) or by the determination of inorganic phosphate release (33). GS kinetic studies were performed using the microtiter assay described in Ref. 33. Analysis of DNA sequences was done by automated sequencing using a PerkinElmer Life Sciences Applied Biosystems 373 DNA sequencer and the Prism dye terminator reaction at the DNA Sequencer Core Facility of the University of Tennessee. Protein analyses were done using the BCA (Pierce Biochemicals) or the Bradford (Bio-Rad) methods and the manufacturer’s protocol.

**RESULTS**

Isolation of a 40-kDa Soybean Nodule Protein Interacting with the C-terminal Domain of Nodulin 26—The nod26 C terminus is composed of a hydrophilic 24-amino acid extension
(Fig. 1), which is exposed to the cytosolic side of the symbiosome (5), and has a sequence that is conserved among group I of nod26-like intrinsic proteins (7). Previous studies (11–13, 16–22) have shown that the C-terminal domain of MIPs is a site for protein-protein interaction. To investigate the possibility that the nod26 cytosolic C-terminal extension serves as a protein interaction site, resins with immobilized synthetic peptides with the nod26 C-terminal sequence were used in affinity chromatography experiments with soybean nodule extracts. Chromatography of a nodule soluble extract on nod26 C-terminal peptide-agarose resulted in the adsorption of a major 40-kDa protein band, which was bound tightly to the resin, requiring 6 M urea for elution (Fig. 1).

**Identification of the 40-kDa Nodulin 26–interacting Protein as Cytosolic Glutamine Synthetase—**The 40-kDa protein band, which was bound tightly to the resin, requiring 6 M urea for elution (Fig. 1B). This was the only detectable protein in the urea eluent. Resolution by two-dimensional electrophoresis revealed that the 40-kDa protein band actually consisted of a collection of bands with distinct pIs and slightly different molecular masses (Fig. 1C). The significance of this two-dimensional electrophoretic profile is discussed below.

Identification of the 40-kDa Nodulin 26–interacting Protein as Cytosolic Glutamine Synthetase—The 40-kDa protein band was excised from an SDS-PAGE gel, digested with trypsin, and subjected to MALDI-TOF mass spectrometry analysis (Fig. 2A). Analysis of the peptide mass fingerprint (Table 1) identified soybean cytosolic glutamine synthetase GS$_1$ as the most likely candidate protein ($E$ value = $6.1 \times 10^{-5}$, 56% sequence coverage). Confirmation of this assignment was obtained by tandem mass spectrometry analysis of a 1610.022-Da tryptic peptide, which yielded a sequence of HKEHIAAYGEG-NER characteristic of soybean GS$_1$. In agreement with the observed 40-kDa molecular mass of the protein on an SDS-PAGE gel (Fig. 1B), identified soybean nodule GS (49 units) was incubated with nod26 peptide-agarose (CK-25-agarose) or with underivatized $\omega$-aminoxyethyl-agarose. The fraction of GS activity bound to the resins (solid bars) as well as in the unadsorbed fraction (open bars) was measured ($n = 6$, error bars show S.E.).

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**FIGURE 1.** Isolation of soybean nodule proteins interacting with the C-terminal domain of nod26. A, topology of symbiotic nod26 in the SM. The conserved NPA loops and the amino acid positions of the ar/R selectivity filter (H2, H5, LE1, and LE2) are indicated based on Wallace et al. (7). The sequence of the C-terminal cytosolic domain is shown with the site of phosphorylation (55) indicated with an asterisk. B, results of affinity chromatography of a soluble extract of 28 day old nodules on the nod26 C-terminal peptide affinity resin. Lane 1, soybean nodule extract (10 $\mu$g of protein). Lane 2, affinity resin-bound protein eluted with 6 M urea. MW, molecular mass markers. C, results of two-dimensional gel electrophoresis of 3 $\mu$g of the affinity resin-purified protein from panel B. The positions of pH markers in the first dimension and molecular mass standards in the second dimension are shown. Lowercase a and b designations show the positions of migration of soybean GS$_1$ and GS$_2$ isoforms, respectively, based on Ref. (23).

**FIGURE 2.** Identification of the 40-kDa protein as soybean cytosolic glutamine synthetase. A, MALDI-TOF spectrum of protonated tryptic peptides (MH$^+$) of the 40-kDa protein isolated by affinity chromatography on nod26 peptide-agarose. The 40-kDa protein was resolved by electrophoresis as in Fig. 1B and subjected to in-gel tryptic digestion and mass spectrometric analysis. The $y$ axis shows the intensity as arbitrary units. The mass-to-charge ratio is plotted on the $x$ axis. The results of mass fingerprinting analysis are in Table 1. B, interaction of GS with the C-terminal domain of nod26. Purified native soybean nodule GS (49 units) was incubated with nod26 peptide-agarose (CK-25-agarose) or with underivatized $\omega$-aminoxyethyl-agarose. The fraction of GS activity bound to the resins (solid bars) as well as in the unadsorbed fraction (open bars) was measured ($n = 6$, error bars show S.E.).
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TABLE 1
Mass fingerprint of 40 kDa protein tryptic peptides from MALDI-TOF spectroscopy

| Measured mass (Da) | Predicted mass (Da) | Residue indices | Sequence |
|-------------------|--------------------|-----------------|----------|
| 692.392           | 692.385 (0.007)     | 219–223         | YILER    |
| 785.382           | 785.334 (0.048)     | 327–332         | DIFEDR   |
| 841.532           | 814.491 (0.041)     | 268–275         | AADKLKG  |
| 1436.042          | 1435.755 (0.287)    | 39–52           | TLPKGPS0PELPK |
| 1610.022          | 1609.759 (0.263)    | 277–290         | HKEHAAYEGNER |
| 1737.602          | 1737.854 (0.252)    | 276–290         | KHEHIHAAYEGNER |
| 1779.212          | 1778.902 (0.310)    | 19–34           | GLNLIVICADYTPAGEAPTPNK |
| 1812.332          | 1812.039 (0.293)    | 224–240         | ITEIAGGGVVSDFPK |
| 1843.212          | 1842.901 (0.312)    | 296–311         | RHHYTPVYVWAAGG |
| 2356.362          | 2356.172 (0.190)    | 85–106          | RNINILVICADYTPAGEAPIPTNK |
| 2512.562          | 2512.273 (0.289)    | 84–106          | GNINILVICADYTPAGEPTAPNK |
| 2668.442          | 2668.374 (0.068)    | 84–107          | RNINILVICADYTPAGEPTAPNK |
| 2946.632          | 2946.479 (0.153)    | 113–137         | VFSHPYVAEVVPNQEEGLYYLQK |
| 2999.612          | 2999.392 (0.220)    | 53–79           | WNYDIGSTGQAPGEDSVEIPYQQARK |
| 3017.392          | 3017.416 (0.023)    | 138–165         | DIQWPLGWPGFPQPGPYPYGCGVGADK |

* The error between the experimental and theoretical masses of each peptide is shown parenthetically.

A, purified recombinant soybean GS1; B, lane 2, purified native soybean nodule GS. Each lane contains 0.5 μg of purified protein. B, fluorescence emission spectra of 0.64 μM NBD-labeled CK-25 in the presence (solid line) or absence (dotted line) of 1.36 μM recombinant soybean GS, β2. λ<sub>e</sub> = 480 nm. C, binding curve of NBD-labeled CK-25 and recombinant soybean GS, β1. The peptide was kept constant at 0.67 μM, and the change in the intensity of fluorescence emission at 545 nm (excitation wavelength = 480 nm) was monitored in response to an increase in the concentration of GS, β1. Error bars show S.E. (n = 4).

To quantify the interaction of the C-terminal nod26 domain with cytosolic GS1/β1, a fluorescence spectroscopy approach was used (Fig. 3). Recombinant GS1/β1 was expressed with an amino-terminal His tag in E. coli and purified by Ni<sup>2+</sup> chelate chromatography (Fig. 3A). The CK-25 peptide containing the full-length soybean nod26 C-terminal domain was labeled on its amino-terminal cysteine with the fluorescent label NBD. In the presence of an equal molar concentration of purified GS1/β1, the labeled CK-25 peptide showed an increase in fluorescence intensity at its emission maximum of 545 nm (Fig. 3B), which was used as an index of peptide-enzyme interaction. The peptide showed saturable binding with half-saturation occurring at a [GS]/[NBD-CK-25] ratio of 0.51, suggesting a binding stoichiometry of one peptide:one GS monomer (Fig. 3C). Assuming 1:1 binding stoichiometry, a fit of the binding data yielded a K<sub>d</sub> of 266 nM (S.E. = 18 nM) for peptide binding to recombinant GS1/β1.

To assay the ability of native nod26 to interact with GS, SMs were isolated from soybean nodules by the Percoll step gradient method, which produces vesicles with the hydrophilic nod26 C terminus exposed on the outer surface of the vesicle (5). Control SMs contained a small but significant amount of GS activity (Fig. 4C). This observation is consistent with previous proteomic analyses that showed that SMs possess peripherally associated GS (34). Incubation of SMs with purified native soybean nodule GS resulted in additional membrane adsorption of GS. Binding of the enzyme to SMs was competitively inhibited by preincubating the purified native soybean nodule GS with 10 μM nod26 peptide (Fig. 4C), suggesting that the nod26 C terminus is responsible for SM binding of GS.

Cytosolic Glutamine Synthetase Interacts with Full-length Nodulin 26 in Vivo—Soybean nodules contain four cytosolic GS isoforms (GS1/β1, GS1/β2, GS1/γ1, and GS1/γ2) that are distinguished by molecular weight and isoelectric point (23). Analysis of the two-dimensional electrophoretic profile in this study (Fig. 1C) suggests that both β and γ isoforms are represented in the GS fraction that binds to the C-terminal nod26 peptide. To investigate the isoform specificity and to test the interaction of full-length nod26 and GS in vivo, a split ubiquitin yeast two-hybrid screen (27) was employed. The full-length cDNAs corresponding to GS1/β1, GS1/β2, GS1/γ1, and GS1/γ2 “preys” were cloned as translational fusions to a modified N-terminal frag-
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The nodule-specific glutamine synthetase (GS) is a critical enzyme in nitrogen assimilation, and its interaction with nodulin 26 (nod26) was studied using the yeast split ubiquitin system. Recombinant forms of both GS1 and GS2 cytosolic soybean nodule GS isoforms were expressed and folded in yeast. Addition of ubiquitin results in the reconstitution of YFP, which is measured using fluorescence microscopy. nod26 was translationally fused to the YFP-C-terminal fragment, which was translated at the amino terminus of GS1 and GS2, resulting in a positive interaction as indicated by YFP fluorescence. The interactions of nod26 with GS1 and GS2 were confirmed using the BiFC (bimolecular fluorescence complementation) assay, which revealed that both GS1 and GS2 proteins interacted with nod26, and the interactions were specific and not due to nonspecific protein-protein interactions.

To determine whether nod26 interacts with GS in planta, BiFC experiments were performed. BiFC is a protein interaction technique in which two putative interacting proteins are fused to either an N-terminal or a C-terminal fragment of yellow fluorescent protein (YFP-N and YFP-C) and are expressed separately. If the two proteins interact, the N- and C-terminal fragments will be brought into close proximity, resulting in the reconstitution of YFP, which can be measured by fluorescence microscopy. nod26 was translationally fused to the YFP-N fragment at either its amino-terminal (YFP-N/nod26) or its carboxyl-terminal (nod26/YFP-C) end. The YFP-C-terminal fragment was translationally fused to the amino-terminal end of GS1 (YFP-C-GS1). The Arabidopsis potassium channel AtKAT1 has also been demonstrated to form oligomers (27). Therefore, the subunit-subunit interactions between monomers of these proteins served as excellent positive controls and were among the most robust interactions reported in this screen (Fig. 4B). The homooligomerization results also suggest that both of these proteins are properly expressed and folded in yeast. Additionally, the wild-type N-terminal fragment of ubiquitin (NubWT) served as a system control because it constitutively interacts with Cub and activates both reporter genes without prey protein attached (Fig. 4B).

Mating of yeast strains containing the four soybean GS isoform prey constructs with strains containing the nod26 bait construct resulted in a positive interaction as indicated by β-galactosidase expression and growth on histidine selection medium (Fig. 4A). As a negative control, an AtKAT1 potassium channel bait construct (27) was used in mating experiments with the GS1 prey constructs. These matings showed no apparent expression of β-galactosidase or growth on histidine selection medium (Fig. 4B). Overall, the data suggest that all four cytosolic soybean nodule GS isoforms form a complex with soybean nod26. This conclusion is supported by the observation that recombinant forms of both β and γ isoforms were bound to isolated SM (supplemental Fig. 2).

To determine whether nod26 interacts with GS in planta, BiFC experiments were performed (Fig. 5). BiFC is a protein interaction technique in which two putative interacting proteins are fused to either an N-terminal or a C-terminal fragment of yellow fluorescent protein (YFP-N and YFP-C) and are expressed separately. If the two proteins interact, the N- and C-terminal fragments will be brought into close proximity, resulting in the reconstitution of YFP, which can be measured by fluorescence microscopy. nod26 was translationally fused to the YFP-N fragment at either its amino-terminal (YFP-N/nod26) or its carboxyl-terminal (nod26/YFP-C) end. The YFP-C-terminal fragment was translationally fused to the amino-terminal end of GS1 (YFP-C-GS1). The Arabidopsis potassium channel factor HY5 has been previously demonstrated to dimerize in BiFC experiments and localize to the nucleus (30), and the Arabidopsis potassium channel AtKAT1 has also been demonstrated to form oligomers (27). Therefore, the subunit-subunit interactions between monomers of these proteins served as excellent positive controls and were among the most robust interactions reported in this screen (Fig. 4B). Overall, the data suggest that all four cytosolic soybean nodule GS isoforms form a complex with soybean nod26. This conclusion is supported by the observation that recombinant forms of both β and γ isoforms were bound to isolated SM (supplemental Fig. 2).

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Plant cytosolic GS1 is encoded by a small, highly conserved gene family in angiosperms (41), with three GS1 isoform classes (des-

The SM is a unique symbiotic interface between legume hosts and endosymbiotic rhizobia bacteria in N₂-fixing symbio-
oses. Biogenesis of the SM occurs early in the rhizobia infection process and is accompanied by the biosynthesis of a variety of

The potential symbiotic significance of nod26 interaction with GS can be understood from the perspective of the known transporters and pumps on the SM (1, 2, 10, 48–52) and the inherent toxicity of ammonia/ammonium transport across energized membranes (53), and it is summarized in Fig. 6. N₂ fixation by rhizobium bacteroids results in the production of NH₃, which diffuses across the bacteroid membrane into the symbiosome space. Efflux of NH₃/NH₄⁺ from the symbiosome space to the cytosol can occur by 1) directional transport of energized membranes (53), and it is summarized in Fig. 6. N₂ fixation by rhizobium bacteroids results in the production of NH₃, which diffuses across the bacteroid membrane into the symbiosome space. Efflux of NH₃/NH₄⁺ from the symbiosome space to the cytosol can occur by 1) directional transport of

by high levels of ammonia (23, 43). The γ isoforms are selectively expressed as nodulin proteins in a developmentally regu-
lated fashion in soybean nodules (23, 44) and other legumes (39, 43, 45). The expression of the four GS1 isoforms during soybean nodule development coincides with the onset of nitrogen fixa-
tion (23), consistent with a role as the major enzymes responsi-
sible for the ATP-dependent assimilation of fixed ammonia transported from the symbiosome to the cytosolic compart-
ment. The expression of GS1 (occurring at approximately day

From a structural perspective, questions remain regarding the composition and binding determinants for the association of the nod26-GS complex. The four soybean GS1 isoforms expressed in nodules share more than 85% amino acid sequence identity (supplemental Fig. 1), and the observation that all interact with nod26 suggests that these proteins contain a con-
served interaction site for the nod26 C-terminal domain. Thus, any isoform could conceivably form a complex with nod26 in vivo. X-ray crystallography of plant cytosolic GS (46) shows a homododecameric structure of two stacked pentameric subunit rings with catalytic sites shared between adjacent monomeric subunits. The finding of a one-to-one binding stoichiometry suggests that each GS monomer possesses a binding site for the nod26 C-terminal domain. The high apparent affinity (Kᵢ = 266 nM) for the interaction of C-terminal nod26 domain for GSβ₁, combined with the observation that both nod26 (as high as 15% of the symbiosome membrane protein (6)) and GS (2% of the nod26 cytosolic protein (47)) are major components of the soybean infected cell, strongly suggest that cytosolic GS binds to the nod26 C terminus at biologically relevant concentrations. This is supported by the yeast split ubiquitin and BiFC experiments.

Analysis of the effects of the nod26 CK-25 peptide on the activity and kinetics of GS (supplemental Fig. 3) suggests that the interaction exhibits a modest effect on enzyme activity with a 25% increase in Vₘₜₐₓ and no significant effect on the Kₘ for NH₄⁺. Although the actual site of interaction on the GS decamer remains to be elucidated, the findings suggest that interaction with nod26 could serve principally to localize GS to the surface of the SM rather than exerting an effect on enzyme activity.

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A diffusive pathway for NH$_3$ efflux also exists with nod26 repressing assimilation of NH$_3$/NH$_4^+$ at the symbiosome surface, which would enhance the rate of fixation with GS would increase the local concentration of the enzyme potentially as a “metabolic funnel” (Fig. 6B). Entry of NH$_4^+$ into the more alkaline plant cytosol would result in a loss of a proton generating NH$_3$, which could reenter the symbiosome space, possibly through nod26, which could represent the lowest energy pathway for NH$_3$ in the SM. The result would be a net transport of a proton from the symbiosome space to the cytosol, which would dissipate the proton motive force generated by the SM H$^+$-ATPase (51) and lead to hydrolysis of ATP and futile cycling. As stated above, the interaction of nod26 with GS could facilitate rapid NH$_4^+$ assimilation, preventing its accumulation in the cytosol. The maintenance of low cytosolic concentrations of NH$_4^+$, which are estimated to be 50-fold lower than the NH$_4^+$ concentration in nitrogen-fixing symbiosomes (54), would prevent potential futile cycling.

Another potential level of complexity in the nod26-GS interaction comes from the observation that both binding partners are subject to post-translational phosphorylation (5, 55–57). In the case of nod26, the unique site of phosphorylation is Ser$^{262}$ (55), which resides in the C-terminal domain, which is the site of GS interaction. Ser$^{262}$ phosphorylation is catalyzed by a calcium-dependent protein kinase that is localized to the SM (5). nod26 phosphorylation is developmentally regulated, becoming apparent at the onset of nitrogen fixation, and is maintained at steady-state levels throughout the N$_2$-fixing portion of the nodule lifespan (9). In addition, phosphorylation is regulated by osmotic stress signals (9), which may reflect the regulation of nod26 transport as part of an osmoregulatory response. Preliminary analyses suggest that nod26 peptides that are phosphorylated at Ser$^{262}$ retain the ability to bind to GS in vitro, but the influence of phosphorylation on the assembly and activity of the complex in vivo remains to be addressed. Cytosolic GS$_1$ is also a target for posttranslational phosphorylation by various protein kinases in plant tissues (56, 57), with phosphorylation potentially leading to interaction with 14-3-3 proteins (56) and other unidentified phosphoproteins (57). The interplay between phosphorylation, GS regulation, and interaction with nod26 and other potential regulatory targets and the effects of these on N$_2$ fixation and assimilation in response to environmental cues remain a topic for future investigation.

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