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NodV and NodW, a Second Flavonoid Recognition System Regulating *nod* Gene Expression in *Bradyrhizobium japonicum*

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In *Bradyrhizobium japonicum*, members of two global regulatory families, a LysR-type regulator, $NodD_1$, and a two-component regulatory system, NodVW, positively regulate *nod* gene expression in response to plant-produced isoflavone signals. By analogy to other two-component systems, NodV and NodW are thought to activate transcription via a series of phosphorylation steps. These include the phosphorylation of NodV in response to the plant signal and the subsequent activation of NodW via the transfer of the phosphoryl group to an aspartate residue in the receiver domain of NodW. In this study, we demonstrated that NodW can be phosphorylated in vitro by both acetyl phosphate and its cognate kinase, NodV. In addition, in vivo experiments indicate that phosphorylation is induced by genistein, a known isoflavone *nod* gene inducer in *B. japonicum*. By using site-directed mutagenesis, a NodWD70N mutant in which the aspartate residue at the proposed phosphorylation site was converted to an asparagine residue was generated. This mutant was not phosphorylated in either in vitro or in vivo assays. Comparisons of the biological activity of both the wild-type and mutant proteins indicate that phosphorylation of NodW is essential for the ability of NodW to activate *nod* gene expression.

Bacteria belonging to the genera *Azorhizobium*, *Rhizobium*, and *Bradyrhizobium* are able to form a root nodule symbiosis with their legume hosts. The establishment of such a symbiosis involves the regulation and expression of the bacterial nodulation (*nod*) genes. In *B. japonicum*, the *nod* genes are induced in response to plant-secreted isoflavone compounds and result in the synthesis of lipochitooligosaccharides, or Nod signals. The Nod signals are able to induce many of the early nodulation responses in plants such as root hair deformation and curling (reviewed in reference 10). The control of *nod* gene expression in response to plant isoflavones appears to be positively regulated by at least two regulatory pathways; the first involves a regulatory protein common to other rhizobia (i.e., $NodD_1$), and the second involves NodVW. $NodD_1$ is a member of the LysR family of transcriptional regulators (18), which include proteins such as *Escherichia coli* LysR, IlvY, and CysB and *Salmonella typhimurium* MetR. Activation of *nod* gene expression by $NodD_1$ involves the binding of the $NodD_1$ protein to the conserved *nod* box sequences present upstream of the respective *nod* operons. Although $NodD_1$ is essential for *nod* gene expression, *B. japonicum* mutants defective in $NodD_1$ were found to be capable of nodulating their host plants, although at a much lower rate than that of the wild type (15, 29). This apparent contradiction was resolved with the subsequent discovery that a second regulatory component, NodVW, is also involved in the activation of *nod* gene expression by isoflavonoids (36). The importance of NodV and NodW had previously been reported by Göttfert et al. (14), who showed that mutations in these genes eliminated the ability of *B. japonicum* to nodulate mung bean, cowpea, and siratro, alternative host plants of *B. japonicum*. In contrast, NodV and NodW are not required for nodulation of soybean. Given these observations, it was postulated that soybean plants may produce isoflavones that can interact with either NodVW or $NodD_1$. Moreover, the requirement for NodV and NodW for nodulation of the alternative host plants could be explained by the possibility that these plants produce inducers that interact specifically with NodVW and not $NodD_1$. Alternatively, this host specificity could be attributed to the levels of Nod signals that are required for the nodulation of the host plants. Efficient nodulation of mung bean, siratro, and cowpea, as compared to soybean, may require levels of Nod signals that are efficiently provided only in the presence of both regulatory proteins.

DNA sequence analyses of *nodVW* indicated that both NodV and NodW are members of the two-component family of regulatory proteins (14). Members of this family can be classified into two main subclasses, the sensor class and the regulator class (reviewed in references 7 and 43). On detecting the appropriate environmental stimulus, the sensor transduces this signal to the regulator, which then regulates the expression of its target genes. These processes are mediated by a series of phosphorylation steps. On receiving the stimulus, the kinase domain of the sensor autophosphorylates itself at a conserved histidine residue. The activated kinase then transfers the phosphoryl group to an aspartate residue in the N-terminal domain of the regulator protein, thereby activating it. In a like fashion, NodV and NodW were suggested to function as the sensor and regulator proteins in the two-component regulation of *B. japonicum nod* genes, regulating gene expression via phosphorylation (14, 36).

In this report, we present data that demonstrate directly that NodW can be phosphorylated in vitro by both acetyl phosphate and its cognate kinase, NodV. This phosphorylation is induced by genistein, an inducer of *nod* gene expression in *B. japonicum*. By site-directed mutagenesis and subsequent phosphorylation assays, we also demonstrate that the conserved aspartate at residue 70 is the likely site of phosphorylation in NodW. Finally, we also demonstrate, using *nod-lacZ* expression and

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
B. japonicum		
USDA 110-573	Wild type, chromosomal *nodC-lacZ* fusion, Tc^r	11
Bj 613-573	*nodW*, Sp^r, Sm^r *nodC-lacZ* fusion, Tc^r	15
E. coli		
JM109	*endA1 recA1 gyrA96 thi hsdR17* (r_K^- m_K^+) *relA1 supE44* λ^-, Δ(*lac-proAB*) [F′, *traD36 proAB lacI*^{q}ZΔM15]	Promega
S17-1	RPR 2-Tc::Mu-Km::Tn*7 pro hsdR recA*	37
Plasmids		
pGEX-4T-3	Ap^r	Pharmacia
pGW11-2	Apr, pGEX-4T-3::*nodW*; NodW protein expressed as fusion with GST	This study
pGWmut-4	Ap^r, pGEX-4T-3; expressing NodWD70N	This study
pGVS1-6	Ap^r, pGEX-4T-3; expressing NodV protein	This study
pGVS4-4	Ap^r, pGEX-4T-3; expressing NodV-truncated protein	This study
pTE3	Tc^r	12
pTE3Km-1	Tc^r, Km cassette	This study
pTE3WWT3	Tc^r, Km^r, pTE3Km-1::*nodW*; wild-type NodW expressed from the *trp* promoter of pTE3	This study
pTE3Wmut10	Tc^r, Km^r, pTE3Km-1; expressing NodWD70N from the *trp* promoter of pTE3	This study
pUC129	Ap^r	22
pUC129W	Ap^r, pUC129::*nodW*	This study
pBSL15	Ap^r, Km^r	2

plant nodulation assays, that *B. japonicum* strains defective in *nodW* can be successfully complemented by the wild-type *nodW* but not by the mutated *nodW* gene encoding a protein (NodWD70N) lacking this phosphorylation site. Thus, phosphorylation of NodW in response to plant-produced isoflavone plays a key role in the modulation of *nod* gene activity in *B. japonicum.*

MATERIALS AND METHODS

Bacterial strains and media. Descriptions of bacterial strains used in this study are listed in Table 1. For routine growth and plant tests, *B. japonicum* strains were cultured on RDY medium (40) at 30°C. *B. japonicum* cells used in mating experiments were grown in HM salt medium (8) supplemented with 0.1% arabinose. Minimal medium (4) was used when β-galactosidase activity assays were conducted. The *E. coli* strains were grown at 37°C in Luria-Bertani medium (34). Antibiotics were used at the following concentrations: kanamycin, spectinomycin, and tetracycline at 100 μg/ml and chloramphenicol at 30 μg/ml for *B. japonicum*; and ampicillin at 50 μg/ml, kanamycin at 40 μg/ml, tetracycline at 25 μg/ml, streptomycin at 30 μg/ml, and spectinomycin at 100 μg/ml for *E. coli.*

Construction of plasmids. A schematic diagram of the plasmids described below is shown in Fig. 1. To express NodV and NodW, the encoding genes were cloned into the plasmid pGEX-4T-3 (Pharmacia, Piscataway, N.J.). This plasmid contains an inducible *tac* promoter and allows for high-level expression of proteins as fusions with the glutathione-*S*-transferase (GST) protein. DNA for cloning was generated by PCR (33) amplification of the NodV and NodW coding regions with primers (Integrated DNA Technologies, Coralville, Iowa) homologous to the 5′ and 3′ ends, hereafter defined as the sense and antisense primers of the respective coding sequences. The template for the PCRs was plasmid

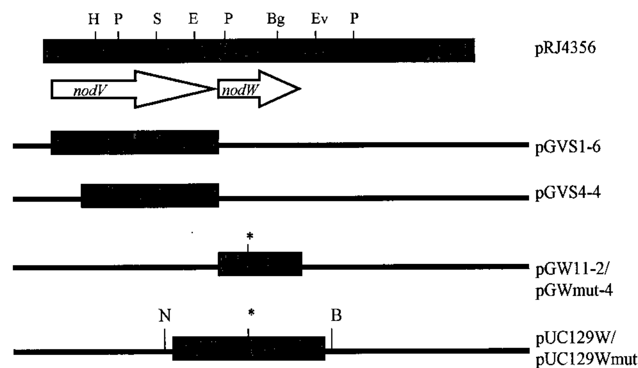

FIG. 1. Restriction map of the *nodVW* region in pRJ4356, and the plasmid constructs used in this study. The restriction map of *B. japonicum* DNA found in pRJ4356 is shown at the top. Only the pertinent restriction sites are shown. Arrows indicate the position of *nodV* and *nodW*. In pGVS1-6, pGVS4-4, pGW11-2, and pGWmut-4, the thick lines represent the pGEX-4T-3 vector into which the coding sequence of the *nodVW* region was cloned. In pUC129W and pUC129Wmut, the thick line represents the pUC129 vector into which the *nodW* sequence was cloned. The asterisk represents the GAT → AAT mutation found on the pGWmut-4 and pUC129Wmut constructs. H, *Hin*dIII; P, *Pst*I; S, *Sal*I; E, *Eco*RI; Bg, *Bgl*II; Ev, *Eco*RV; N, *Nsi*I; B, *Bam*HI.

pRJ4356 (14). Contained within the primers were nucleotide sequences that would result in the generation of either *Bam*HI or *Not*I sites at the 5′ and 3′ ends of the PCR products, respectively. The NodW sense primer was 5′-GCGGATC CGTGACAGGACGATTTGA-3′, and the antisense primer was 5′-ATGCGG CCGCTCATACTTGGGTTTG-3′. For the cloning of NodV, two separate PCRs were performed, resulting in the synthesis of either the full-length *nodV* sequence or a truncated *nodV* that contains a deletion of 414 nucleotides (1 to 414) at the 5′ end of *nodV*. This deletion resulted in the removal of sequences encoding the predicted hydrophobic spanning region of NodV. The sense primers used in the PCRs were 5′-TTGGATCCGGTCTCAAAATGTATAG-3′ (full-length *nodV*) and 5′-TTGGATCCGCAATTTTCGAGCA-3′ (truncated *nodV*). The same antisense primer, 5′-ATGCGGCCGCTCCAGTCAAATCGTC-3′, was used in both reactions. Following the PCRs, the PCR-amplified fragments were cloned into the *Bam*HI and *Not*I site of pGEX-4T-3. The resultant *nodV* and *nodW* plasmids were sequenced by the dideoxy chain termination method of Sanger et al. (35). DNA sequence analysis was used to confirm the in-frame fusions of NodW or NodV with GST and to show that no mutations had been generated by the PCRs. As a result, plasmid pGW11-2 was chosen as an authentic NodW clone while plasmids pGVS1-6 and pGVS4-4 were selected to express the full-length and truncated forms of NodV, respectively.

The NodW mutant plasmid pGWmut-4 was constructed by site-directed mutagenesis of pGW11-2 with the USE (unique-site elimination) mutagenesis kit (Pharmacia). The synthetic primer (5′-dGCTGTAGTACTTAATGTCCGGCT GC-3′) used for the mutagenesis reactions contained a single base mutation, GAT to AAT, that resulted in conversion of the aspartate (D) at residue 70 to asparagine (N). In addition, the mutagenic oligonucleotide harbored a second, but neutral mutation (TCT to ACT) 4 nucleotides upstream of the AAT codon that results in the addition of a *Sca*I site in plasmids that have incorporated the mutagenic primer. Clones arising from the mutagenesis reactions were initially screened for the presence of the *Sca*I site. Putative mutants were then sequenced, and pGWmut-4, containing the desired mutation, was chosen to express NodWD70N.

Plasmids pTE3WWT-3 and pTE3Wmut10 were generated to express wild-type NodW and NodWD70N, respectively, from the *trp* promoter of the broad-host-range vector pTE3Km-1. Plasmid pTE3Km-1 was constructed by insertion of the kanamycin resistance cassette of pBSL15 (2) into the *Bgl*II site of pTE3 (12). pTE3WWT-3 and pTE3Wmut10 were constructed as follows. Plasmid pRJ4356 was digested with *Sal*I and *Eco*RV, and the 2.3-kb fragment containing *nodW* and portions of the 3′ end of *nodV* were ligated into the *Sal*I-*Sma*I site of pUC129 (22), creating pUC129W (Fig. 1). A corresponding plasmid encoding NodWD70N was also constructed by digesting pGWmut-4 with *Pst*I-*Bgl*II and exchanging the 0.5-kb *Pst*I-*Bgl*II fragment containing the D70N mutation with the corresponding *Pst*I-*Bgl*II fragment of pUC129W. Plasmids pUC129W and pUC129Wmut were subsequently digested with *Nsi*I and *Bam*HI, and the *nodW* fragments were cloned into the *Pst*I-*Bam*HI site of pTE3Km-1, creating pTE3WWT-3 and pTE3Wmut10, respectively. The resulting plasmids were then transformed into *E. coli* S17-1 (37) and mobilized by biparental mating (3) into different *B. japonicum* strains that contained a chromosomally encoded *nodC-lacZ* fusion. Transconjugants were selected on the basis of resistance to kanamycin.

Purification of NodV and NodW. An overnight culture of JM109 (50 ml) harboring either pGW11-2, pGWmut-4, pGVS1-6, or pGVS4-4 was used to inoculate 2.5 liters of Luria-Bertani broth containing 50 μg of ampicillin per ml. When the culture had reached a cell density of approximately 5×10^8 cells/ml, isopropyl-β-D-thiogalactopyranoside (IPTG; Boehringer Mannheim, Indianapolis, Ind.) was added to a final concentration of 100 μM, and the culture was grown for a further 1.5 h. The cells were then collected by centrifugation and resuspended in phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulfonyl fluoride and 1 μg each of leupeptin, pepstatin, DNase I, and chymostatin (Sigma, St. Louis, Mo.) per ml. Cell lysis was carried out by incubation with lysozyme (1 mg/ml) on ice for 20 min followed by sonication on ice for six 30-s cycles with a Sonifier model 450 (Branson Ultrasonics, Danbury, Conn.) at full power. Cell debris was removed by centrifugation (30,000 × *g* for 30 min), and the supernatant was applied to a glutathione-agarose column (Sigma). After extensive washing with PBS, specific cleavage of the *nod* gene product from the GST domain of the fusion protein was achieved by passage of the site-specific protease thrombin through the column. This makes use of the property that the recognition sequence for thrombin is encoded immediately upstream of the fusion cloning site in pGEX-4T-3. The eluted material, containing both the *nod* gene product and an additional two amino acids (Gly and Ser) from the thrombin cleavage site, was then analyzed by silver staining (5) after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (23). For nomenclature purposes, the purified proteins containing these two extra amino acids are designated NodV (when pGVS1-6 is used), truncated NodV (when pGVS4-4 is used), NodW (when pGW11-2 is used), and NodWD70N (when pGWmut-4 is used). The protein concentration was determined with the bicinchoninic acid (BCA) reagent kit (Pharmacia), using bovine serum albumin (BSA) as a standard. Fractions containing the *nod* gene products were dialyzed individually against PBS containing 50% glycerol and stored in aliquots at −20°C.

Antibody reagents. Fractions containing NodW were concentrated by acetone precipitation. The NodW fractions were mixed in ice-cold methanol at a ratio of 4:1 (vol/vol) and incubated in a dry ice-methanol bath for 30 min before being centrifuged at 30,000 × *g* (16). The pellet was resuspended in 2× SDS sample buffer and analyzed by Coomassie brilliant blue staining after SDS-PAGE. Gel slices containing NodW were specifically excised, and the NodW protein was electroeluted by the method of Harlow and Lane (17). The NodW samples (~1 mg) were concentrated by ultrafiltration in a Centricon-10 concentrator (Amicon Inc., Beverly, Mass.), homogenized with Freund's complete adjuvant (Sigma), and injected into a female New Zealand White rabbit. Booster injections of 0.5 mg in Freund's incomplete adjuvant (Gibco Laboratories, Grand Island, N.Y.) were administered every 4 weeks. Blood from the rabbit was collected 1 week after each boost. The antiserum was collected and stored at −20°C in 1-ml aliquots. For further purification of NodW antibodies, a NodW affinity column was prepared by coupling purified NodW to a cyanogen bromide (CNBr)-activated agarose column (Sigma), using the method described by the manufacturer. Anti-NodW antiserum was fractionated over the column, and the anti-NodW antibodies were eluted with 0.1 M glycine (pH 2.3). The anti-NodW fractions were then dialyzed against PBS and stored in 1-ml aliquots at −20°C.

Phosphorylation of NodW by acetyl [^{32}P]phosphate. Acetyl [^{32}P]phosphate was prepared as described by Stadtman (42) with [^{32}P] orthophosphate (Dupont NEN, Boston, Mass.). The amount of acetyl phosphate was quantitated by colorimetric assay with a known amount of acetyl phosphate (Sigma) as a standard (38, 42). A specific activity of 50 mCi/mmol was obtained. For the phosphorylation reactions, proteins (30 pmol) were incubated at 25°C for 1, 5, or 10 min in 15 μl of buffer containing 50 mM Tris · HCl (pH 7.0), 2 mM $MgCl_2$, 0.1 mM dithiothreitol, and 10 mM acetyl [^{32}P]phosphate. The reactions were terminated at the indicated times by the addition of 15 μl of 2× SDS sample buffer (120 mM Tris · HCl, 4% SDS, 20% glycerol, 0.002% bromophenol blue) containing 50 mM EDTA (SDS termination buffer). The samples were placed on ice and analyzed by SDS-PAGE (12% polyacrylamide). Following electrophoresis, the gels were exposed to X-Omat X-ray film (Eastman Kodak, Rochester, N.Y.) for autoradiography.

In vitro phosphorylation assays for NodV and NodW. The phosphorylation assays were carried out at room temperature in 30 μl of phosphorylation buffer containing 50 mM HEPES (pH 8.0), 50 mM KCl, 5 mM $MgCl_2$, and 2 mM EDTA. The phosphorylation reactions were initiated by the addition of 25 μM [γ-^{32}P]ATP or [α-^{32}P]ATP (ICN Pharmaceuticals, Costa Mesa, Calif.) and terminated at various time points with SDS termination buffer. To test the phosphorylation of NodW by NodV, the NodV proteins (5 pmol) were incubated in the phosphorylation assay mixture for 15 min. NodW (30 pmol) was then added, and the reaction was stopped at various time points by the addition of SDS termination buffer. The samples were then analyzed by autoradiography after SDS-PAGE.

In vivo phosphorylation of NodW. In vivo phosphorylation assays were performed by a modification of a method described by Karr et al. (21). *B. japonicum* cells were cultured in HM medium for 3 days and then transferred to minimal medium buffered with 3-(*N*-morpholino)propanesulfonic acid (MOPS; Sigma) and containing limiting phosphate (0.06 g/liter). At an absorbance at 600 nm of 0.2 (approximately 72 h after subculture), the cultures were induced by the addition of genistein to a final concentration of 2 μM. A 0.5-mCi portion of [^{32}P]orthophosphate (ICN Pharmaceuticals) was added to give a final concentration of 10^7 cpm/μmol of phosphate, and the cultures were grown for a further 4 h. The cells were pelleted, washed in minimal medium, and resuspended in 1 ml of lysis solution (20 mM Tris [pH 7.5], 10 mM EDTA, 1% SDS). The samples were boiled, and the cell extracts were clarified by centrifugation. NodW proteins were then immunoprecipitated by the method of Stader et al. (41). Briefly, 20 μl of cell extracts was mixed with 0.65 ml of immunoprecipitation buffer (50 mM Tris [pH 8.0], 0.15 M NaCl, 10 mM EDTA, 2% Triton X). To this mixture, 10 μl of anti-NodW (200 μg/ml) was added, and the samples were incubated overnight at 4°C. A 50-μl volume of protein A-Sepharose (Pierce, Rockford, Ill.) was then added, and the samples were incubated for an additional 1 h at 4°C. The immunoprecipitate was pelleted by centrifugation, washed twice with 50 mM Tris (pH 8.0) containing 10 mM EDTA, resuspended in SDS sample buffer, and boiled for 5 min. Protein A-Sepharose was then removed by centrifugation, and the supernatant was subjected to SDS-PAGE. The polypeptides were transferred to an Immobilon-P membrane (Millipore, Bedford, Mass.), and the radiolabeled protein was visualized by autoradiography. Following autoradiography, NodW antigens were detected by immunoblotting with anti-NodW antibody and alkaline phosphate-conjugated goat anti-rabbit secondary antibody (1:3,000). Immunoreactive material was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Pierce) as substrates.

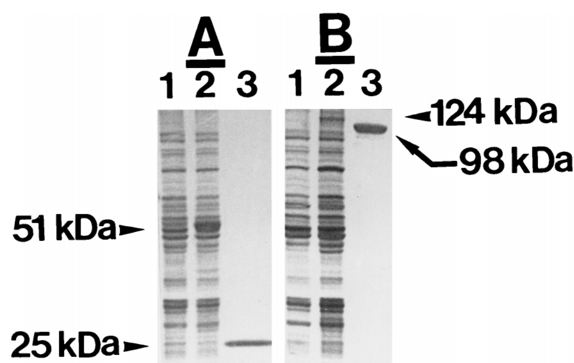

FIG. 2. Purification of NodV and NodW. NodW (A) and NodV (B) were purified, as described in Materials and Methods, from *E. coli* extracts harboring pGW11-2 and pGVS1-6, respectively. Lanes: 1, cell extract; 2, IPTG-induced cell extracts; 3, purified protein.

β-Galactosidase assays. β-Galactosidase activity was assayed as described by Banfalvi et al. (3). The induction of *nod-lacZ* expression by either genistein (2 μM) or soybean seed extract was performed as described by Yuen and Stacey (46). Soybean seed extract was prepared as described by Smit et al. (39).

Plant nodulation assays. Seeds of *Glycine max* (soybean) cv. Essex and *Vigna radiata* (mung bean) cv. King were surface sterilized as described by Nieuwkoop et al. (29). After germination for 48 h, the seedlings were transferred to plastic growth pouches (Mega International, Minneapolis, Minn.). Two days after the transfer, each seedling was inoculated with 10^8 cells of the appropriate bacterial culture. Plants were grown in a growth chamber with a 16-h photoperiod. The plants were watered, as necessary, with a nitrogen-free nutrient solution (44). The plants were examined every 3 days for nodule formation beginning 7 days postinoculation.

RESULTS

Purification of NodV and NodW. By using the purification method described in Materials and Methods, the majority of NodW and NodWD70N was fractionated into the soluble fraction and could be purified directly by chromatography over a glutathione-agarose column. SDS-PAGE analysis and subsequent silver staining of the thrombin-eluted fraction revealed a distinct band of 25 kDa, which corresponded to the expected molecular mass of NodW (Fig. 2A, lane 3) (data not shown for NodWD70N). Typically, a yield of 2 mg of protein per 10 liters of culture was obtained. Attempts to purify NodV in a similar fashion did not meet with the same success, because the majority of NodV protein synthesized was found in the insoluble fraction. NodV purification therefore required an initial solubilization of the inclusion bodies with 1 mM CHAPS (3-[(3-cholamindopropyl)dimethylammonio]-1-propanesulfonate) (Sigma). The solubilized proteins were dialyzed extensively against PBS, and the dialysate was applied to a glutathione affinity column. The NodV proteins were then eluted by thrombin treatment.

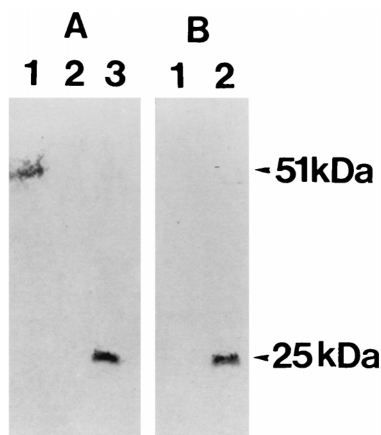

FIG. 3. Immunoblot analyses with anti-NodW. (A) Anti-NodW antibody was used to probe cell extracts of IPTG-induced *E. coli* harboring pGW11-2 (lane 1) or pGEX-4T-3 (lane 2). Purified NodW was loaded in lane 3. (B) Labeling of *B. japonicum* cell extracts. Lanes: 1, strain Bj613 (*nodW−*); 2, strain USDA110.

The purified NodV and the truncated NodV proteins migrated with molecular weights of 98,000 and 83,000, respectively, on SDS-PAGE gels (Fig. 2B, lane 3) (data not shown for the truncated NodV). These polypeptide sizes were in good agreement with the predicted molecular masses of 98 and 83 kDa for NodV and NodV (truncated) respectively.

Antibodies directed against NodW. Polyclonal antibodies were generated against NodW in New Zealand White rabbits. The affinity-purified antibody, designated anti-NodW, labeled a single band of 51 kDa in extracts from IPTG-induced *E. coli* cultures containing plasmid pGW11-2 (Fig. 3A, lane 1). The size of this labeled protein is consistent with the predicted size of the NodW-GST fusion (25-kDa NodW and 26-kDa GST). As the antibody did not react with extracts of *E. coli* expressing only the GST protein (lane 2), the labeling of this 51-kDa polypeptide can be attributed specifically to the reactivity of the antibody to the NodW polypeptide. When anti-NodW was used to probe cell extracts of *B. japonicum* USDA110, the antibody labeled a polypeptide of 25 kDa, the expected size for NodW (Fig. 3B, lane 2). In contrast, no labeling of cell extracts from the NodW mutant strain Bj613 was observed (lane 1).

In vitro phosphorylation of NodW with acetyl [^{32}P]phosphate. As previous reports on several bacterial response regulator proteins had demonstrated that the regulator components could be phosphorylated by small-molecule phosphodonors (e.g., acetyl phosphate, phosphoramidate, or carbamoyl phosphate [25]), the ability of NodW to be phosphorylated by acetyl phosphate was similarly tested. NodW protein was incubated in the phosphorylation buffer containing 2 mM $MgCl_2$ and 10 mM acetyl [^{32}P]phosphate. As shown in Fig. 4, the purified NodW protein incorporated ^{32}P within 5 min of incubation in the presence of acetyl [^{32}P]phosphate. NodW samples pretreated with 10 mM EDTA prior to the addition of acetyl phosphate demonstrated no detectable phosphorylation pattern. This result is consistent with the observation that Mg^{2+} is required for phosphorylation to occur. The negative control, BSA, was not phosphorylated.

NodV is autophosphorylated in the presence of [γ-^{32}P]ATP. Phosphorylation of NodV was tested by incubating the purified protein in 5 mM $MgCl_2$ and 20 μM either [γ-^{32}P]ATP or [α-^{32}P]ATP. As indicated in Fig. 5, addition of NodV to the phosphorylation buffer containing [γ-^{32}P]ATP resulted in detectable levels of NodV phosphorylation within 15 s of the initiation of the reactions. A steady increase was observed up to 15 min following incubation. In contrast, NodV was not phosphorylated in the presence of [α-^{32}P]ATP (data not shown). The above phosphorylation pattern was also observed with the truncated NodV (data not shown).

FIG. 4. In vitro phosphorylation of NodW by acetyl [^{32}P]phosphate. BSA or purified NodW (30 pmol) was incubated in phosphorylation buffer (50 mM Tris.HCl [pH 7.0], 2 mM $MgCl_2$, 0.1 mM dithiothreitol) containing 10 mM acetyl [^{32}P]phosphate in the presence or absence of 10 mM EDTA. Incubations were done for 5 min (odd-numbered lanes) or 10 min (even-numbered lanes). The reactions were terminated at the indicated times by the addition of SDS termination buffer, and the proteins were separated in an SDS–15% polyacrylamide gel. Lanes: 1 and 2, BSA; 3 and 4, BSA plus EDTA; 5 to 8, NodW plus EDTA; 9 and 10, NodW.

NodW is phosphorylated by NodV. To determine whether NodV can phosphorylate NodW, NodV was incubated with [γ-^{32}P]ATP for 15 min. NodW was then added to the ^{32}P-NodV reaction mixture or alternatively to [γ-^{32}P]ATP, and the samples were incubated for a further 15 min. When NodW was incubated with [γ-^{32}P]ATP alone, no NodW phosphorylation could be detected (data not shown). In contrast, NodW phosphorylation could be detected as early as 30 s after the start of the incubation with ^{32}P-NodV. Increasing lengths of incubation with ^{32}P-NodV protein resulted in increasing levels of phospho-NodW (Fig. 6A). A corresponding decrease in the level of ^{32}P-NodV was also observed, with essentially little or no ^{32}P-NodV detected at 15 min, suggesting that the phosphate group of ^{32}P-NodV was directly transferred to the NodW protein.

Aspartate 70 is essential for NodW phosphorylation. Sequence comparisons of NodW to other regulator proteins indicated that aspartate residue 70 is the likely phosphorylation site of NodW. Studies of several regulator proteins (6, 20, 24, 28) have shown that an aspartate-to-asparagine conversion of this residue was sufficient to prevent phosphorylation of the regulator protein. Site-directed mutagenesis was thus performed with a target primer that replaces the Asp70 codon (i.e., GAT) with an asparagine codon (i.e., AAT). As shown in Fig. 6B, incubation of NodWD70N with ^{32}P-NodV showed no labeling of the regulator protein, with most of the radioactive phosphate remaining with the sensor kinase NodV. This result supports the notion that the Asp70 residue is the site of phosphorylation on NodW.

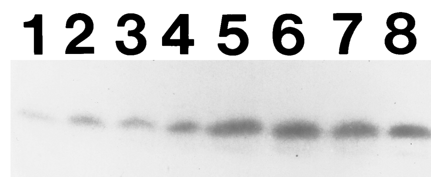

FIG. 5. Autophosphorylation of NodV. NodV was incubated for increasing times in phosphorylation buffer, as indicated in Materials and Methods. Reactions were terminated with SDS termination buffer and analyzed by autoradiography after SDS-PAGE. Lanes: 1, 0.25 min; 2, 1 min; 3, 2 min; 4, 5 min; 5, 10 min; 6, 15 min; 7, 20 min; 8, 30 min.

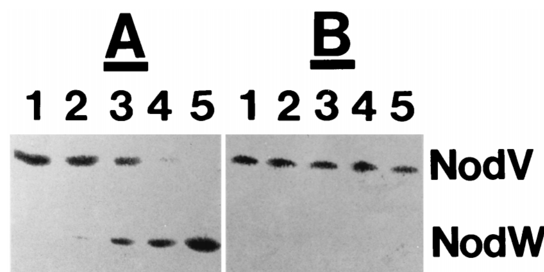

FIG. 6. Time course of NodW phosphorylation by NodV. Purified NodV was incubated with [γ-^{32}P]ATP for 15 min as described in Materials and Methods. NodW was then added, and the reactions were terminated at various time points with SDS termination buffer. The samples were run on SDS–15% acrylamide gels, and the radioactivity associated with the polypeptides was visualized by autoradiography. (A) NodW; (B) NodWD70N. Lanes: 1, 0 min; 2, 0.25 min; 3, 0.5 min; 4, 1 min; 5; 5 min.

In vivo phosphorylation is induced by genistein. Previous results have indicated that NodW is essential for isoflavonoid-induced expression of the *nodYABC* and *nodD$_1$* operons (36). As a consequence, NodV and NodW were proposed to specifically interact with isoflavonoid compounds (e.g., genistein), leading to the expression of the *nod* genes under their control. Given that the phosphorylation cascade observed in the two-component regulatory systems is evoked in response to environmental stimuli, the effects of genistein treatment on NodW phosphorylation was tested. For this purpose, plasmids expressing NodW (i.e., pTE3WWT3) and NodWD70N (i.e., pTE3Wmut10) were mobilized into the *nodW* mutant strain Bj613. Transconjugants expressing the wild-type NodW [i.e., Bj613(pTE3WWT3)] or NodWD70N [i.e., Bj613(pTE3W mut10)] were treated with genistein, and the respective cell extracts were incubated with anti-NodW to immunoprecipitate the NodW proteins. Analysis of the immunoprecipitates revealed detectable levels of ^{32}P-NodW only in the Bj613(pTE3WWT3) sample that had been treated with genistein (Fig. 7B). Consistent with the in vitro phosphorylation data, no phosphorylation of NodWD70N from Bj613 (pTE3Wmut10) was observed. Subsequent immunoblot analysis with anti-NodW (Fig. 7A) ensured that the observed results were not a consequence of unequal loading of the NodW proteins. These results support the notion that in vivo phosphorylation of NodW is induced by the flavonoid genistein.

Biological importance of NodW phosphorylation. To characterize the biological significance of NodW phosphorylation

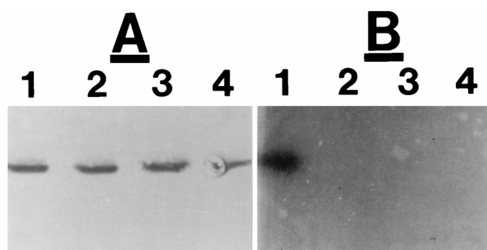

FIG. 7. In vivo phosphorylation of NodW. *B. japonicum* cells were labeled as described in Materials and Methods. NodW proteins were then immunoprecipitated from cell extracts, and the immunoprecipitated material was separated by SDS-PAGE and transferred to an Immobilon-P membrane. ^{32}P-labeled polypeptides were revealed by autoradiography. Following autoradiography, NodW antigens were detected by immunoblotting with anti-NodW and goat anti-rabbit alkaline phosphatase. (A) Immunoblot; (B) autoradiograph. Lanes: 1 and 2, Bj613(pTE3WWT3); 3 and 4, Bj613(pTE3Wmut10); 1 and 3, plus genistein; 2 and 4, no treatment.

TABLE 2. Comparison of *nodC-lacZ* expression in *B. japonicum* strains USDA110 and Bj613 complemented with either wild-type NodW (i.e., pTE3WWT3) or NodWD70N (i.e., pTE3Wmut10)

Strain and plasmid	Activity (β-galactosidase units)[a] with following inducer:		
	None	Genistein	SSE
USDA110-573			
No plasmid	7 ± 1	437 ± 15	764 ± 61
pTE3Km-1	13 ± 2	450 ± 61	683 ± 34
pTE3WWT-3	68 ± 6	882 ± 85	2,852 ± 83
pTE3Wmut10	10 ± 1	228 ± 17	327 ± 34
Bj613-573			
No plasmid	7 ± 1	6 ± 0	7 ± 1
pTE3Km-1	7 ± 1	4 ± 1	5 ± 1
pTE3WWT-3	56 ± 8	743 ± 36	2,011 ± 93
pTE3Wmut10	7 ± 1	7 ± 1	18 ± 1

[a] These units are with chlorophenol red–β-galactopyranoside (CPRG) as a substrate. The results are the mean values of two independent determinations; each determination was done in three replicates.

on *nod* gene expression, pTE3WWT3 and pTE3Wmut10 were mobilized individually into the *nodW* mutant Bj613 strain that contains a chromosomally encoded *nodC-lacZ* fusion (i.e., Bj613-573) (14). The resultant strains were tested for the ability to mediate *nod* gene induction by both isoflavonoids and soybean seed extracts (SSE). Comparisons of *nod* gene expression revealed little or no *nod* gene induction by genistein or SSE in either the Bj613-573 or the Bj613-573 strain harboring the NodW mutant pTE3Wmut10 construct (Table 2). In contrast, Bj613-573 strains harboring the wild-type NodW construct [i.e., Bj613-573(pTE3WWT3)] showed a dramatic increase in *nod* gene expression when induced by both genistein and soybean seed extract. A parallel analysis was also carried out with *B. japonicum* wild-type strains harboring the above plasmids. Transconjugants of USDA110 harboring pTE3WWT-3 [i.e., USDA110(pTE3WWT3)] demonstrated levels of *nod* gene expression that were significantly higher than those obtained with USDA110-573. This increase was detected in both uninduced and induced cultures. In contrast, *nod* gene levels were approximately twofold lower in the strains expressing NodWD70N [i.e., USDA110(pTE3Wmut10)] than in the untransformed USDA110-573 strain.

The biological effects of Asp70 phosphorylation were further tested by plant infection tests of mung bean and soybean roots. In these assays, the nodulation abilities of Bj613-573 strains, harboring a plasmid encoding either wild-type NodW or NodWD70N, were compared to determine if the nodulation phenotype of strain Bj613-573 could be complemented by expressing these proteins. The controls were *B. japonicum* USDA110-573 or Bj613-573 complemented with vector alone. As shown in Table 3, no significant difference in the nodulation phenotype on soybean was observed with the various transconjugants (both USDA110-573 and Bj613-573), except for a slight delay in nodulation in strain Bj613-573 complemented with the vector alone. However, parallel examinations of the nodulation phenotype on mung bean revealed significant differences. For instance, no nodules were detected in the Bj613-573 strain complemented with either vector [i.e., Bj613-573(pTE3Km-1)] or pTE3Wmut10, even 21 days postinoculation. In contrast, strain Bj613-573(pTE3WWT3) complemented with NodW resulted in a nodulation phenotype similar to that observed with the wild-type inoculum (i.e. [USDA 110(pTE3Km-1)].

TABLE 3. Nodulation phenotype of *B. japonicum* 613 (*nodW*) strains transformed with either pTE3WWT3 (expressing NodW) or pTE3Wmut10 (expressing NodWD70N)

Strain	No. of nodules on day after Inoculation[a]:							
	Mung bean				Soybean			
	7	10	13	21	7	10	13	21
USDA110-573 (pTE3Km-1)	0	1 ± 1	4 ± 1	8 ± 2	3 ± 1	7 ± 1	15 ± 2	17 ± 2
Bj613-573 (pTE3Km-1)	0	0	0	0	0	2 ± 1	11 ± 2	14 ± 1
Bj613-573 (pTE3WWT3)	0	1 ± 1	4 ± 4	9 ± 2	2 ± 1	8 ± 2	14 ± 3	17 ± 2
Bj613-573 (pTE3Wmut10)	0	0	0	0	2 ± 1	4 ± 1	13 ± 2	15 ± 2

[a] Nodule numbers per plant were compared at 7, 10, 13, and 21 days after inoculation. The results presented are the means of two trials with nine plants per trial. Standard errors are given.

DISCUSSION

In previous studies, *nodW* was shown to play a key role in the regulation of *nod* gene expression and in the ability of *B. japonicum* to infect its host plants (14, 36). Sequence analyses revealed that both NodV and NodW share homology with members of a family of two-component regulators that form an important paradigm for signal transduction in bacteria (7, 43). The proteins found in these families are of two subclasses, the sensor and regulator classes. In response to environmental stimuli, the sensor proteins modulate the activity of the regulator proteins by a phosphotransfer reaction. In this study, we detail several lines of evidence that indicate that both NodV and NodW are phosphorylated and that phosphorylation is essential not only for *nod* gene expression but also for the nodulation of mung bean by *B. japonicum*. For example, NodW can be phosphorylated in vitro by acetyl phosphate. Small-molecule phosphodonors such as phosphoaramidate, carbamoyl phosphate, and acetyl phosphate have been reported to function in vitro in place of the sensor kinases to phosphorylate purified regulator proteins, resulting in the activation of these proteins (13, 25, 27). Consistent with other sensor kinases, purified NodV can also be autophosphorylated in vitro by [γ-^{32}P]ATP. This in vitro phosphorylation was observed in the absence of genistein (see below) and suggests that purification of NodV from its presumed membrane localization may result in conformational changes in NodV that obviate the requirement for the inducer. Phosphorylated NodV is capable of phosphorylating NodW in vitro. In contrast, a mutant form of NodW, NodWD70N, lacking the Asp70 residue, cannot be phosphorylated. In NodWD70N, the aspartate residue at position 70 was replaced by an asparagine residue. Similar mutations, resulting in a null phenotype, have been reported in other regulator proteins (e.g., CheYD57N, VirGD52N, and NtrCD54N) (6, 20, 24, 28). The availability of the nonphosphorylated NodWD70N allowed us to test the biological significance of phosphorylation for both *nod* gene expression and nodulation.

Previously, we reported that NodW is required for the induction of *nod* gene expression in the presence of plant-produced isoflavonoids. For example, genistein-induced expression of *nodY-lacZ* and *nodD$_1$-lacZ* fusions was drastically reduced in a *B. japonicum* strain mutated for *nodW* when compared to that in the wild type (36). As a consequence, it was proposed that plant-specific signals were perceived by NodV and that this signal was then transduced via the phosphorylation of NodW. To evaluate the significance of NodW phosphorylation on *nod* gene expression, plasmids encoding either the wild-type NodW or NodWD70N were mobilized into strain Bj613-573 or USDA110-573 harboring a chromosomal *nodC-lacZ* fusion. Expression of NodWD70N was unable to phenotypically complement the *nodW* mutation in Bj613-573, as indicated by the lack of induced expression of the *nodC-lacZ* fusion in response to genistein or SSE treatment. In contrast, the expression of wild-type NodW restored the inducible expression of the fusion in this strain. Consistent with these results, we also demonstrated that NodW is phosphorylated in vivo upon the addition of genistein. Similar to the observations obtained in the in vitro studies, no phosphorylation of NodWD70N was detected. Our present results are consistent with those observed for other two-component systems in which phosphorylation of the regulator proteins is induced in response to some form of environmental stimuli (e.g., acetyosyringone-induced phosphorylation of VirG [20]). A likely model for *nod* gene induction by flavonoids would therefore involve the flavonoid-induced phosphorylation of NodW, at aspartate 70, leading to the subsequent mediation of *nod* gene activity by phospho-NodW.

In addition to the above results, several other interesting observations were made when the *nodC-lacZ* fusions were analyzed. For example, expression of NodW from a constitutive promoter, in the absence of genistein, resulted in a fivefold increase in the basal expression of the *nodC-lacZ* fusion in both USDA110 and Bj613 backgrounds. Given our present observation that NodW phosphorylation is induced by genistein, a possible explanation for this observation could be that unphosphorylated NodW can by itself function as a transcriptional activator of *nod* genes. In this connection, it has been reported that the unphosphorylated forms of several response regulators (e.g., PhoB and OmpR) are still able to initiate transcription (19, 26). Phosphorylation, in these cases, has been shown to increase the in vitro transcriptional activity of the regulators. In the case of OmpR, for example, this increase in transcriptional activity appears to occur as a result of increased DNA binding ability of the phosphorylated form of OmpR to its target promoter DNA (1). As our present results reveal little or no increase in the basal levels with the corresponding strains expressing NodWD70N, this rationalization would also imply that the role of the aspartate 70 residue extends beyond that of being the site of phosphorylation of NodW. An alternative explanation for the increase in the basal levels of *nod* gene expression is the possibility that a small fraction of NodW is phosphorylated even in the uninduced state. These levels are, however, not sufficient to activate the target genes above background. Constitutive expression of NodW would result in increased amounts of phospho-NodW and a subsequent increase in the basal level of *nod* gene activity. Consistent with this, no increase in *nod* gene activity with strains expressing NodWD70N is observed.

A second interesting observation was that USDA110-573 strains expressing NodWD70N from a *trp* promoter of pTE3

Wmut10 resulted in lower *nod* gene activity than in USDA110-573 strains. Given the mechanism by which the two-component regulator proteins have been reported to function, several possibilities may explain the inhibitory effect of the mutant protein on *nod* gene expression. For instance, the active form of NodW may be a dimer, and the formation of heterodimers of NodW and NodWD70N may result in inactive complexes, thus causing phenotypic suppression. The requirement for dimerization has been reported in the regulation of the *ntr* genes by NtrC (30, 31, 45). Phosphorylation of the NtrC receiver domain increases the cooperativity of binding of dimers to two (enhancer) sites on the target DNA. This oligomerization of dimers is essential for the activation of transcription (30). On the other hand, NodW may function as a monomer. In this case, competition by NodWD70N for the same promoter sites may result in less of the active NodW binding to the promoter sequences, leading to lower *nod* gene activity. This second possibility appears to be more likely, because sequence analyses indicate that NodW is most similar to the FixJ class of regulator proteins (14, 43), which appear to function as monomers. Both the phosphorylated and unphosphorylated forms of FixJ have been demonstrated to bind to their target promoter DNAs, with phosphorylation of FixJ stimulating the transcription of its target genes (32). Phosphorylation in the N-terminal receiver domain is proposed to alleviate the repressive effect that the N-terminal domain of FixJ has on the protein effector (i.e., C-terminal) domain.

In addition to determining the effect of phosphorylation on *nod* gene activity, the effects of phosphorylation of NodW on the ability of *B. japonicum* to nodulate its plant host were also analyzed. Previously, Göttfert et al. (14) had shown that *nodW* was essential for the nodulation of cowpea, siratro, and mung bean, alternative host plants of *B. japonicum*. However, NodW was not necessary for the nodulation of soybean. The results of the nodulation assays correlate well with the previous nodulation data and are consistent with the levels of *nod* gene expression observed in the respective strains. For instance, the *B. japonicum* strain mutated for *nodW* was able to nodulate soybean but not mung bean. Complementation of this strain with the wild-type NodW but not NodWD70N restored not only *nod-lacZ* expression but also the ability of the mutant strain to nodulate mung bean. These results further support the notion that phosphorylation of NodW at Asp70 is required for its function as a transcriptional activator of *nod* gene expression in vivo.

In conclusion, our present results support the view that the regulation of *nod* gene expression by NodV and NodW involves phosphorylation. Thus, in *B. japonicum*, there are two possible modes for the recognition of the flavonoid inducers of *nod* gene expression. The first comprises a common mechanism found among rhizobial species, involving NodD. The second entails the isoflavonoid-induced phosphorylation and activation of the *nod* gene regulator NodW. This involvement of multiple regulatory proteins in *nod* gene induction in response to plant stimuli has also been observed in other rhizobia. For example, the control of *nod* genes in *Rhizobium meliloti* involves four LysR-type proteins, namely, $NodD_1$, $NodD_2$, $NodD_3$ and SyrM (9). These proteins activate the transcription of the *nod* genes in response to different plant signals, hence providing the bacteria with a means of modulating the production of Nod signals in response to changes in the plant environment. In the case of *B. japonicum*, multiple LysR-type proteins do not appear to be present to interact specifically with different flavonoid signals. Instead, *B. japonicum* uses a two-component regulatory system, in which in the presence of the plant isoflavonoid, phosphorylation is the mechanism used to regulate *nod* gene expression. The use of phosphorylation thus provides an alternative pathway, different from that of the LysR regulators, by which *nod* gene activation by flavonoids occurs. The unique involvement of both NodW and $NodD_1$ in the positive regulation of the *Bradyrhizobium nod* genes does raise several questions. For instance, does NodW (phosphorylated and unphosphorylated) interact with the *nod* promoters? Given that $NodD_1$ binds to the *nod* box sequences of the *nod* promoters, it will be interesting to determine if the target site for NodW binding is similar to that of $NodD_1$. Also, the interaction of NodW (unphosphorylated and phosphorylated) and $NodD_1$ proteins remains a possibility. It will be of interest to determine whether $NodD_1$ interacts independently with the *nod* promoters or perhaps in a cooperative manner with the activating NodW protein.

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