Modulating DNA bending affects NodD-mediated transcriptional control in *Rhizobium leguminosarum*

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ABSTRACT

*Rhizobium leguminosarum* NodD binds to the *nod* box of the inducible *nod* gene *nodA* as a V-shaped tetramer and bends the *nod* box. In this work, we show that the *nod* gene inducer naringenin decreased gel mobility of *nod* box DNA–NodD complexes by sharpening the NodD-induced DNA bend, which correlated with *nodA* transcription activation. NodD can induce different DNA bends when the distance between the two half-sites of the *nod* box was modified, which severely affected NodD-mediated transcriptional control. One or two base pairs were deleted from, or inserted into, the two half-sites of the *nod* box of *nodA*. Circular permutation assays showed that such distance modulations allowed NodD to induce relaxed or sharpened DNA bending. In the case of 1 bp deletion, where the DNA bends were more relaxed than in the wild type, *nodA* transcription was repressed both in the absence and in the presence of inducer naringenin. In the cases of 1 and 2 bp insertion, where the DNA bends were much sharper than in wild type in the absence or presence of the inducer naringenin, *nodA* transcription was initiated constitutively with no requirement for the inducer naringenin or, even, the NodD regulating protein.

INTRODUCTION

Symbiosis between rhizobia and leguminous plants under condition of nitrogen starvation leads to the development of nitrogen-fixing nodules, in which the bacteria reduce air N₂ into ammonia. The nodulation (nod) genes of rhizobia, which are organized in several operons located either on the chromosome or on large (Sym) plasmids, play an important role in the development of nodules. The expression of many inducible *nod* genes is positively regulated by the trans-activator NodD. This protein binds to the conserved *cis*-regulatory element *nod* box preceding *nod* operons and upon interaction with the inducing flavonoid activates transcription of these operons (1–5). However, the exact mechanism of how NodD responds to inducer to initiate transcription of inducible *nod* gene is not fully understood.

NodD is a member of the LysR-type transcriptional regulators (LTTRs), which constitute one of the largest regulating families in prokaryotes, which activate transcription of their target genes in response to internal or external signal stimuli (6). Many LTTR-controlled regulons are functionally involved in the basic material and/or energetic metabolism, such as amino acid biosynthesis, CO₂ fixation, nitrogen assimilation and catabolism of aromatic compounds (7–9). However, some LTTR-controlled regulons are involved with the specific functions, such as synthesis of virulence factors, signal molecules or response to cold and osmotic stress (10–12). Another feature of LTTRs is that they repress their own transcription (6,13,14).

LTTRs are usually thought to have at least two binding sites, one for a small signal molecule and another for a DNA target (15,16). These small molecules generally do not greatly affect the binding affinity of LTTRs to their DNA targets. DNase I footprinting experiments show that LTTRs usually protect their target promoters approximately from position −75 to −25 relative to the transcriptional start site of their target genes (17–20). The long recruiting site contains two half-sites, one from position −75 to −50, the other from −50 to −25, each harboring an individual LTTR-binding half-site. When bound to DNA, LTTRs induce a DNA bend, whose center is between the two half-sites (21–23).

*Rhizobium leguminosarum* NodD binds to its target DNA as a homo-tetramer (24). The overall crystal structure of a full-length LTTR CbnR has also been reported to form a homo-tetramer and can be regarded as a dimer of dimers, whereby each dimer is composed of two subunits in different conformations, and each subunit has two domains, a DNA-binding
domain and a regulatory domain (25). These results support the view that the tetramers serve as the biologically active form of the LysR family. As a tetramer, the main bodies of CbnR and NodD are, respectively, proven and proposed be V-shaped (24,25). The DNA-binding domains are located at the bottom of the V-shaped main body, suitable to interact with a 60 bp long stretch of the promoter DNA. Interactions between the four DNA-binding domains and the two binding sites on the target DNA are likely to bend the target DNA along the V-shaped bottom of the tetramer (24,25).

The exact mechanism underlying the inducer-triggered LTTR-mediated transcriptional regulation is not fully understood. Recently, several clues indicate that the transcriptional activation may involve the DNA structural modulation by LTTRs (22,23,26,27). The first clue is that the ligand of OccR, octopine, can relax the OccR-induced DNA bend in vitro (22). The DNA bending by CatR is also reported to respond to the inducer cis–cis-muconate (23). The second clue is that the wild-type OxyR-wt causes a sharper DNA bend on the OxySRS promoter than on the positive mutant OxyR-C199S, which is locked in the activated conformation (27). The third clue is that mutations of the inverted repeat of the C199S, which is locked in the activated conformation (27).

The inducer naringenin decreases gel mobility of the nod box DNA–NodD complexes by sharpening a NodD-induced DNA bend (22) after digestion with the same enzymes. Each resulting plasmid was digested with one of the enzymes BglII, XhoI, EcoRV, Smal or BamHI, generating five sets of equal-length fragments with different 5′ and 3′ ends. These fragments were end-labeled and incubated with cell extracts from the R. leguminosarum strain 8401(pIJ1518), which is the R. leguminosarum strain 8401 harboring the plasmid pIJ1518, which is a derivative of the broad host range vector pKT230 and contains a cloned nodD gene from R. leguminosarum, in the presence or absence of 40 μM naringenin and size-fractionated at room temperature using 20 × 15 × 0.8 cm 5% polyacrylamide gels in buffer described previously (24). After electrophoresis, the gel was dried and subjected to autoradiography.

**RESULTS**

The inducer naringenin decreases gel mobility of the wild-type nodA nod box DNA–NodD complexes by sharpening a NodD-induced DNA bend

The conserved nod box of the typical inducible nod promoters is from approximately −25 to −75 relative to the

**PARTIAL DRAWING**

**MATERIALS AND METHODS**

**Microbiological techniques**

Bacterial strains and plasmids are listed in Table 2 or in the text. Media and general growth conditions were as described by Hu et al. (14). Diparental conjugation was performed to mobilize broad host range plasmids from *Escherichia coli* to *R. leguminosarum* as described by Simon et al. (28).

**Enzymes and chemicals**

Restriction endonucleases and DNA ligase were purchased from Promega; [γ-32P]dATP was from Amersham; HiFi-Bst DNA polymerase was produced in our own laboratory (29); other chemical reagents were above analytical grade.

**Plasmid construction**

Using the oligonucleotide EADI and one of the oligonucleotides P-2D, P-1D, PADI, P+1D or P+2D as primers and the plasmid pUCWZ, which is the pUC19 derivative carrying the wild-type nodD-nodA promoter (30) as template, PCR fragments of wild-type nodA promoter and its mutants were produced. These fragments were cloned into the EcoRI–PstI sites of the plasmid pMP221, which is a derivative of and has

an opposite multi-cloning site from the IncP broad-host-range plasmid pMP220 (30,31). The lacZ gene of both pMP21 and pMP220 lacks its native promoter, so these nodA promoter derivatives were fused to the reporter gene lacZ (Table 2). The resulting clones were sequenced (Figure 1 and Table 2). The sequences of the oligonucleotides used as primers are listed in Table 1.

These DNA fragments were also fused to the lacZ gene of the plasmid pMP220 in a similar way to determine whether such different nodA promoter mutations have any effects on NodD-mediated repression of nodD (Table 2).

**DNA bending by circular permutation assay**

Using the oligonucleotides XNODAD1 and SNODAD1 as primers and five plasmids harboring the wild-type or mutant nodA promoter derivatives, namely, pMPD, pMP-2D1, pMP-1D, pMP+1D6 and pMP+2D3 as templates, five PCR amplification products containing the DNA region protected by NodD were synthesized. The lengths of each PCR product were 138, 136, 137, 139 and 140 bp respectively; each containing a SalI site and an XbaI site at their extreme left and right ends, respectively. They were cleaved with these two restriction endonucleases and ligated to the plasmid pBend3 (22) and then transformed into *E. coli*.

**RESULTS**

The inducer naringenin decreases gel mobility of the nod box DNA–NodD complexes by sharpening a NodD-induced DNA bend

The conserved nod box of the typical inducible nod promoters is from approximately −25 to −75 relative to the
transcriptional start site. The most critical base pairs for NodD binding to nod box are 2-fold imperfect inverted repeat with the basic sequence ATC-N9-GAT-N16-ATC-N9-AAT (31,33) (Figure 1).

As mentioned above, R.leguminosarum NodD binds to its target DNA as a homo-tetramer. Inactivation of the nod box distal half-site allows NodD to partially activate nodA transcription in an inducer-independent manner in vivo and sharpens the NodD-induced DNA bending in the absence of inducer in vitro (24). Such LTTRs as OccR, CatR and OxyR relax DNA bending on their target promoters when activating transcription. NodD might change DNA bending when activating transcription. We used the plasmid pBend3 (22) to perform circular permutations. These are based upon the observation that the mobility of a DNA fragment is less when a bend is located at its center than when the same bend is located toward one of its ends (34). pBend3 contains a large number of restriction endonuclease cleavage sites arranged in two tandem sets, with unique sites at the center to introduce the fragment of interest. A 138 bp DNA fragment containing the wild-type nod box of nodA was introduced into this plasmid. The resulting plasmid was individually digested with each of the five different endonucleases, BglII, XhoI, EcoRV, SmalI or BamHI, creating fragments that had the same 241 bp sequence in a permuted order. These fragments were end-labeled and incubated with cell extracts from the R.leguminosarum strains 8401(pIJ1518), which contains the cloned nodD of R.leguminosarum in the presence or absence of 40 µM naringenin. The fragments were then subjected to electrophoresis and autoradiography.

As shown in Figure 2, NodD–DNA complexes formed in both conditions exhibited a strong position-dependent mobility. Such large alterations are generally interpreted as being due to a DNA bend (34). Complexes formed in the presence of naringenin showed a little stronger position-dependent mobility (Figure 2), indicating that naringenin partially sharpened this bend. All complexes that formed in the presence of naringenin migrated more slowly than the equivalent complexes formed in the absence of naringenin (Figure 2).

The bend angles can be estimated using the empirical relationship \( \mu \phi = \cos(\alpha/2) \), where \( \mu_\phi \) is the mobility of the protein–DNA complex with a bend at the center of the DNA fragment, \( \mu_\phi \) is the mobility of the protein–DNA complex with a bend at the end of the DNA fragment and \( \alpha \) is the angle by which the DNA departs from linearity (35). To estimate the bend angle, the distance migrated was plotted against the number of nucleotides separating the middle of nod box
from the left end of each DNA fragment. Using the mobilities of the slowest- and fastest-migrating complexes as data, this equation predicts that complexes without naringenin have a 44 bend, while complexes containing naringenin have a 48 bend. However, the fast-migrating fragment has its bend center far away from either end (about 34 bp), while the slowest-migrating fragment has its bend center somewhat far from the middle (about 11 bp). Therefore, assuming that this equation is valid for the gel system used in this study, these calculated bend angles probably underestimate the true values.

**Distance modulation between the two half-sites of the nod box of nodA changed NodD-induced DNA bending**

We investigated the effects of modulating the nod box on the extent of NodD-induced bending by deleting from or inserting into 1 or 2 bp the proximal and distal half-sites of the nod box of nodA (Figure 1) and performing circular permutation assays to detect the changes of DNA bending (Figure 3).

Using the methods described above, four DNA fragments with lengths of 136, 137, 139 or 140 bp, containing nod box in the constructed nodA promoter derivatives, pMP-2D1, pMP-1D, pMP+1D6 and pMP+2D3, respectively (Figure 1), were introduced into the plasmid pBend3. Each resulting plasmid was digested with BglII, XhoI, EcoRV, SmalI or BamHI, to generate four sets of equal-length fragments, which were end-labeled and incubated with cell extracts from 8401(pIJ1518) in the presence or absence of 40 μM naringenin. Each complex exhibited strong position-dependent mobility, and the mobilities of the complex of the 2 or 1 bp deletion nodA promoter derivatives were much faster than that of wild type, and much faster than that of 1 or 2 bp insertion both in the absence and in the presence of naringenin. The bend angles estimated using the empirical relationship \( \mu_{\text{nod}}/\mu_0 = \cos(\alpha/2) \) revealed that the NodD-induced DNA bend angle of 1 bp deletion nodA promoter derivatives was more relaxed than that of wild type, and more relaxed than that of 1 and 2 bp insertion both in the absence and in the presence of naringenin (Figure 3).

Surprisingly, the complex in case of 2 bp deletion migrated much faster on the gel than the wild type (Figures 2 and 3A), indicating that NodD induced a more relaxed DNA bend on the nod box. However, the estimated bend angles were similar to that of the wild type.

It is also notable that in cases of 1 bp deletion and insertion, all complexes formed in the presence of naringenin migrated more slowly than the equivalent complexes formed in the absence of naringenin. In case of 2 bp deletion, complexes formed in the presence of naringenin migrated similarly to or slightly more slowly than the equivalent complexes formed in the absence of naringenin. However, in case of 2 bp insertion, complexes formed in the presence of naringenin migrated slightly faster than the equivalent complexes formed in the absence of naringenin (Figure 3).

The binding of NodD to these duplexes was qualitatively characterized in an electrophoretic gel mobility shift assay under conditions of stoichiometric binding to test whether such distance modulations between the two half-sites of nodA nod box lead to affinity change of NodD to the nod box (Figure 4). DNA fragments of nodA promoter derivatives whose sequences were listed in Figure 1, namely, pMPD, pMP-2D1, pMP-1D, pMP+1D6 and pMP+2D3, respectively, which represented the wild-type control, 2 bp deletion, 1 bp deletion, 1 bp insertion and 2 bp insertion mutants of nodA promoter, were end-labeled and titrated against protein extracts from the _R. leguminosarum_ strain 8401(pIJ1518) in the absence of naringenin. The binding of NodD to the wild-type nodA promoter DNA was stronger than to the 1 and 2 bp deletion (Figure 4A–D). The binding of NodD to the wild-type nodA promoter DNA was also stronger than to the 1 and 2 bp insertion in the absence of naringenin (Figure 4E–H).

**The artificial DNA bend modulation severely affects the NodD-mediated transcriptional control**

To study the relation between NodD-induced DNA bend with the transcription activation of inducible nod genes, different
nodA promoter derivatives were fused to the lacZ gene of the plasmid pMP221, which is a derivative of and has an opposite multi-cloning site from the IncP broad-host-range plasmid pMP220. The lacZ gene of both pMP221 and pMP220 lacks its native promoter (30,31). These mutant plasmids, and the wild-type control, were each transferred by conjugation into two R. leguminosarum strains, namely 8401(pKT230), which lacks nodD, or its derivative harboring pIJ1518, which contains the cloned nodD of R. leguminosarum. Transconjugants were grown with or without the inducer naringenin and were assayed for β-galactosidase activity. In the case of wild-type control as in pMPD, NodD activated nodA transcription in response to naringenin. In the case of 2 bp deletion as in pMPD-1D, NodD activated nodA transcription in response to inducer naringenin, but the transcription was 4-fold lower when in the presence of naringenin, and 4-fold higher when in the absence of naringenin than that of the wild type under equivalent condition. In the case of 1 bp deletion as in pMP-1D, the transcription of nodA was similar to or slightly higher than that of the wild type in the absence of naringenin and lower than the wild type in the presence of naringenin. In the cases of 1 and 2 bp insertion as in pMP+1D6 and pMP+2D3, nodA transcription was initiated constitutively with no requirement for the inducer naringenin or, even, the NodD regulating protein (Table 3).

To determine whether such different nodA promoter mutations have any effects on NodD-mediated repression of nodD transcription, these DNA fragments were also fused to the lacZ gene of the plasmid pMP220, which has an opposite multi-cloning site from pMP221, then transferred them by conjugation into the two R. leguminosarum strains 8401(pKT230) and 8401(pIJ1518), respectively. The transcriptional levels of the nodD gene were determined by measuring the β-galactosidase activity of these transconjugants grown in the absence of the inducer naringenin. The results are summarized in (Table 3, transcription of NodD-mediated repression). In the case of 2 bp deletion, the transcription of nodD was 4-fold that in the case of wild type both in the presence and in the absence of naringenin. In the case of 1 bp deletion and insertion, the transcription of nodD in the R. leguminosarum strain 8401(pIJ1518) was slightly higher than that in the case of wild type while lower than that in 2 bp deletion. In the case of 2 bp insertion, the transcription of nodD in the R. leguminosarum strain 8401(pIJ1518) was slightly higher than that in the case of wild type as well as 1 bp deletion and insertion but lower than that in 2 bp deletion. The transcription of nodD in the R. leguminosarum strain 8401(pKT230), which lacks NodD among each case, was significantly different.

**DISCUSSION**

So far the exact mechanism of how NodD responds to inducer to initiate transcription of inducible nod gene is not fully understood. Fisher and Long (21) have revealed that NodD binds to two distinct sites on the same face of the helix and induces a bend in the DNA. Our previous studies also suggested that the NodD–nod box might undergo severe trans-formational change when the inducible nod genes initiate transcription (30).

Recently, Shin et al. (25) and Feng et al. (24) have both proposed similar structural models of the LTTR–DNA complex that CbnR or NodD binds to their target DNA as a tetramer, the main body of which is V-shaped. Unlike the DNA targets of many known transcriptional regulators, those of LTTRs are 2-fold imperfect palindromic sequences, containing two half-sites (17–20). Through anchoring the two half-sites of their promoters, the V-shaped LTTRs can bend and twist their DNA targets (24,25).
Small signal molecules are thought to directly interact with LTTRs, and such specific interactions are expected to cause changes, e.g. oligomerization or conformational change, in the LTTR proteins. Several in vitro experiments indicate that multiple LTTRs undergo conformational change rather than oligomerization in response to small signal molecules (16,22, 26,27,36), but the exact role of the conformational changes is not clear. It is possible that they allow direct protein–protein contact between LTTRs and other proteins involved in transcription regulation (37). Another possible mechanism
Although such distance modulation between the two half-sites requires the inducer naringenin (Figure 3 and Table 3), there was no obvious relationship between the severe transcription changes of nodA and the transcription of nodA. Further studies showed that when the distance between the two half-sites of the nodA promoter of 2 bp deletion, 1 bp deletion mutants and the wild-type control, respectively. As for the exact role of the NodD-induced DNA bend, it is possible that the bend has some direct effect upon transcription, but it could be the case that it is simply to allow the regulator protein to contact RNA polymerase at the promoter. Figure 5 presents a model summarizing these findings. The NodD tetramer anchors the two half-sites of the nodA promoter derivatives. The cell extracts from 8401(pIJ1518) incubated in the absence of naringenin used in (H) is 10-fold greater than that in (G) and 10-fold greater than that in (F) and 10-fold greater than that in (E).

Table 3. Transcription of nodA promoter derivatives (A) or NodD-mediated repression of itself (B) as determined by measuring β-galactosidase activity

| Class                      | Plasmid            | Bend angle (°) | +naringenin | Units β-galactosidase | nodA promoter derivatives |
|----------------------------|--------------------|----------------|-------------|------------------------|---------------------------|
| nodA promoter derivatives  |                    |                |             |                        |                           |
| I: Wild type               | pMPD               | 44             | 48          | 83                     | 8401(pKT230)              |
| II: 2 bp deletion          | pMP-2D1            | 44             | 49          | 93                     |                           |
| III: 1 bp deletion         | pMP-1D             | 42             | 30          | 116                    |                           |
| IV: 1 bp insertion         | pMP+1D6            | 55             | 53          | 2443                   |                           |
| V: 2 bp insertion          | pMP+2D3            | 70             | 67          | 2319                   |                           |

| NodD-mediated repression of itself |          |                |             |                        |                           |
| I: Wild type                 | PMP220D         |                |             |                        |                           |
| II: 2 bp deletion            | PMP220-2D1      | 453            | 547         | 555                    |                           |
| III: 1 bp deletion           | PMP220-1D       | 938            | 152         | 267                    |                           |
| IV: 1 bp insertion           | PMP220+1D6      | 350            | 138         | 167                    |                           |
| V: 2 bp insertion            | PMP220+2D3      | 188            | 196         | 272                    |                           |

Plasmids containing nodA promoter derivatives were present in 8401(pKT230) (nodD+) or 8401(pIJ1518) (nodD−). β-galactosidase activity is expressed in Miller Units (U). Naringenin (10 μM) was used as induction condition. Assays of β-galactosidase activities were performed in triplicate and were reproducible within 15% from experiment to experiment. In the transcription of NodD-mediated repression, these DNA fragments were fused to the lacZ gene of the plasmid pMP220, which has an opposite multi-cloning site from pMP221 employed in transcription of nodA promoter derivatives.

Plasmids containing nodA promoter derivatives were present in 8401(pKT230) (nodD+) or 8401(pIJ1518) (nodD−). β-galactosidase activity is expressed in Miller Units (U). Naringenin (10 μM) was used as induction condition. Assays of β-galactosidase activities were performed in triplicate and were reproducible within 15% from experiment to experiment. In the transcription of NodD-mediated repression, these DNA fragments were fused to the lacZ gene of the plasmid pMP220, which has an opposite multi-cloning site from pMP221 employed in transcription of nodA promoter derivatives.
polymerase to form active transcriptional open complex upon the inducible nod gene.

In most cases, the mutation had effects on the bend angle (Figures 2 and 3). However, in some cases, 2 bp deletion mutation had no effect on bend angle. Because according to the empirical relationship $\mu_n/\mu_e = \cos(\alpha/2)$, the angle is determined by the ratio between $\mu_n$ and $\mu_e$. In this particular case, the complex of which migrated much faster on the gel than the wild type (Figures 2 and 3), indicating that NodD induced a more relaxed DNA bend on it, but the value of $\mu_n/\mu_e$, thereby the estimated bend angles showed little difference from that of wild type. The true values of the bend angle in this case need to be further determined employing other methods. Also, it cannot be excluded that factors other than distance influence the mobility of protein-bound fragments. It is notable that in most cases, except in the case of 2 bp insertion, all complexes formed in the presence of naringenin migrated more slowly than the equivalent complexes formed in the absence of naringenin (Figures 2 and 3). The reason for this exception is not clear. In the case of pMP-2D1, nodA transcription triggered differently from that of the wild type, although the calculated DNA bend angles were similar in both cases in the presence and absence of naringenin. This may further indicate that other factors in addition to DNA bending are involved in the control of nod gene transcription (Table 3).

The transcription of nodD of these mutants in 8401(pIJ1518) was slightly or significantly higher than that of wild type. It seems that it is changes in binding affinity of NodD to the nod box that affect the transcription of nodD (Table 3 and Figure 4) and that NodD-directed DNA bend plays a less important role in the regulation of nodD transcription (Table 3 and Figure 4), which confirm our earlier findings that NodD represses its own transcription by competing with RNA polymerase for binding sites (14). The transcription of nodD in 8401(pKT230) among each case was significantly different (Table 3). Perhaps it is for the reason that the binding affinity of RNA polymerase to the promoter of each mutant was different.

There is accumulating evidence that the nod gene expression, hence Nod signal abundance and quality are tightly regulated, affecting nodulation efficiency and specificity (1). There may be additional regulator protein besides NodD involved in the transcription regulation of nod genes. Our hypothesis is reinforced by the fact that a repressor of nodulation genes, NolR, was identified in several different Rhizobium species and was shown to bind to the nodD1–nodA promoter of Sinorhizobium meliloti (1,38,39). The sequence of the repressor NolR of S.meliloti is 78% identical to the sequence predicted from the R.leguminosarum genome sequence currently located at the Sanger web site (http://www.sanger.ac.uk/Projects/R_leguminosarum). Recently, we cloned and sequenced a gene named hurl from R.leguminosarum bv. viciae coding for the HU-like protein Hurl, which was previously observed to bind specifically to nod promoters and be involved in in vitro nodD transcription (4,40,41). Inactivation of hurl led to severe impairment in the nodD expression, repression in the inducible expression of nodA and nodF. These results suggested that hurl might be required for maintaining the normal expression of nod genes in R.leguminosarum bv. viciae (41). Moreover, such protein playing important roles in the global and basic biological events as the molecular chaperone groEL has been reported may take part in the transcription regulation of nod gene (42). These proteins together with NodD and RNA polymerase may form complex and elaborate network in the transcription regulation of inducible nod gene. But the exact mechanism needs further research.

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