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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 N_2 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, CO_2 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

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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 *nod* box distal half-site allow NodD to activate *nodA* transcription in an inducer-independent manner *in vivo*, and to modulate the DNA bending of the NodD–*nod* box complex in the absence of inducer *in vitro* (24).

In an effort to understand the roles that the inducer naringenin and the DNA conformation change play in the transcriptional activation of inducible *nod* genes, and to gain insights into the mechanism of NodD-mediated transcription initiation of inducible *nod* gene, we describe the physical properties of NodD–*nod* box complexes and modulated the distance between the two half-sites of *nodA nod* box, and report the resulting DNA bend angles, binding affinity of NodD to the *nod* box and NodD-mediated transcriptional control both in the presence and in the absence of naringenin.

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; [α-^{32}P]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 pMP221 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* transcription (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) after digestion with the same enzymes. Each resulting plasmid was digested with one of the enzymes BglII, XhoI, EcoRV, SmaI 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* strains 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.

Binding affinity of NodD to the *nod* box

Cell extracts from the *R.leguminosarum* strain 8401(pIJ1518) incubated in the absence of naringenin were used in the titration test in gel mobility shift assays to determine whether modulating the distance between the two half-sites of *nod* box affects the binding affinity of NodD to the *nod* box. The methods were described previously (24).

β-Galactosidase assay

R.leguminosarum strains were incubated at 28°C under aeration in TY medium till the A_{600} value increased to 0.4. Naringenin was added to a final concentration of 10 μM as the induction condition. Assays of β-galactosidase activities were performed in triplicate and were reproducible within 15% from experiment to experiment (32).

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

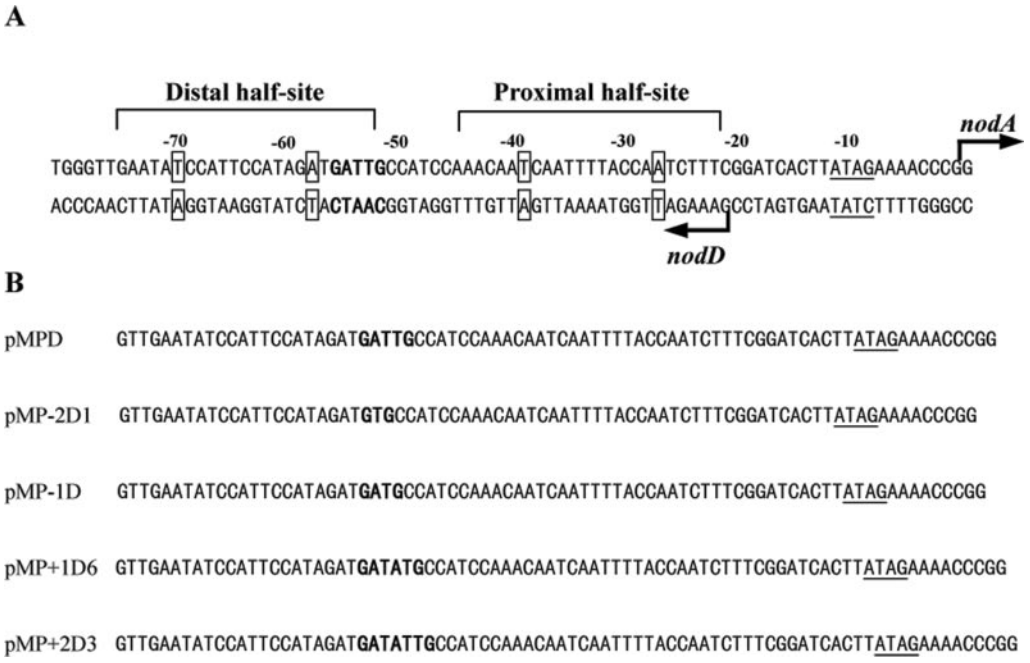

Figure 1. Sequences of inducible wild-type *nodA* promoter and mutant *nodA* promoter derivatives used in this work. (**A**) The inducible wild-type *nodA* promoter of the symbiotic plasmid pRL1JI. Arrows indicate the transcriptional start sites of *nodA* and *nodD*. The transcriptional start site of *nodA* is numbered +1 (14,31). The bases T and A of the putative LysR motif T-N_{11}-A are boxed, indicating the two binding centers of the canonical *nod* box (14,21). The sequences of the conserved –10 are underlined. (**B**) Alignment of inducible wild-type *nodA* promoter and mutant *nodA* promoter derivatives. pMPD is the wild-type *nodA* promoter. The mutant *nodA* promoter derivatives are those with 1 or 2 bp inserted into the wild-type *nodA* promoter between positions −54 and −53 or deleted from positions −54 and/or −55. The left end of each constructed *nodA* promoter derivatives is as shown and the right end of each is located at position +57 relative to the transcriptional start point of *nodA*.

Table 1. Oligonucleotides used as primers in PCR

Oligonucleotide	Sequence
EADI	5′-TCTGAATTCGGGCCCCTGCCCGGC-3′
PADI	5′-ACGCTGCAGTTGAATATCCATTCCATAG-3′
P-2D	5′-TCTGCAGGTTGAATATCCATTCCATAGATGTGC-CATCCAAACAATCAATTTTAC-3′
P-1D	5′-TCTGCAGGTTGAATATCCATTCCATAGATGATG-CCATCCAAACAATCAATTTTAC-3′
P+1D	5′-TCTGCAGGTTGAATATCCATTCCATAGATGATN-TGCCATCCAAACAATCAATTTTAC-3′
P+2D	5′-TCTGCAGGTTGAATATCCATTCCATAGATGATN-NTGCCATCCAAACAATCAATTTTAC-3′
XNODAD1	5′-TCTTCTAGAGGGCCCCTGCCCGGCGCTTC-3′
SNODAD1	5′-ACGGTCGACTTGAATATCCATTCCATAG-3′

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-N_9-GAT-N_{16}-ATC-N_9-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, SmaI 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 $\mu_m/\mu_e = \cos(\alpha/2)$, where μ_m is the mobility of the protein–DNA complex with a bend at the center of the DNA fragment, μ_e is the mobility of the protein–DNA complex with a bend at the end of the DNA fragment and α 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

Table 2. Bacterial strains and plasmids

Strains or plasmids	Relevant characteristics	Reference or source
Rhizobium		
8401	*R.leguminosarum* cured of its symbiotic plasmid; Str^r	(43)
E.coli		
S17-1	294 *recA*, chrom, RP4 derivative	(28)
Plasmids		
pUCWZ	pUC19 derivative carrying the wild-type *nodD–nodA* promoter	(30)
pKT230	IncQ broad-host-range plasmid; Str^r, Kan^r	(44)
pIJ1518	1.8 kb BclI fragment with *R.leguminosarum nodD* in pKT230; Kan^r	(43)
pMP220	*lacZ* downstream of a multi-cloning site; IncP broad-host-range plasmid; Tc^r	(31)
pMP221	Opposite multi-cloning site; pMP220 derivative	(30)
pMPD	pMP221 derivative harboring wild-type *nodA* promoter with the sequence near position −54 GATTG	This study
pMP-2D1	pMP221 derivative harboring mutant *nodA* promoter with the sequence near position −54 GTG, AT deleted	This study
pMP-1D	pMP221 derivative harboring mutant *nodA* promoter with the sequence near position −54 GATG, T deleted	This study
pMP+1D6	pMP221 derivative harboring mutant *nodA* promoter with the sequence near position −54 GATA**T**G, A inserted	This study
pMP+2D3	pMP221 derivative harboring mutant *nodA* promoter with the sequence near position −54 GATA**TT**G, TT inserted	This study
PMP220D	pMP220 derivative harboring wild-type *nodA* promoter with the sequence near position −54 GATTG	This study
PMP220-2D1	pMP220 derivative harboring mutant *nodA* promoter with the sequence near position −54 GTG, AT deleted	This study
PMP220-1D	pMP220 derivative harboring mutant *nodA* promoter with the sequence near position −54 GATG, T deleted	This study
PMP220+1D6	pMP220 derivative harboring mutant *nodA* promoter with the sequence near position −54 GATA**T**G, A inserted	This study
PMP220+2D3	pMP220 derivative harboring mutant *nodA* promoter with the sequence near position −54 GATA**TT**G, TT inserted	This study

from the left end of each DNA fragments. 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 fastest-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, SmaI 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 $\mu_m/\mu_e = \cos(\alpha/2)$ 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 fast 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

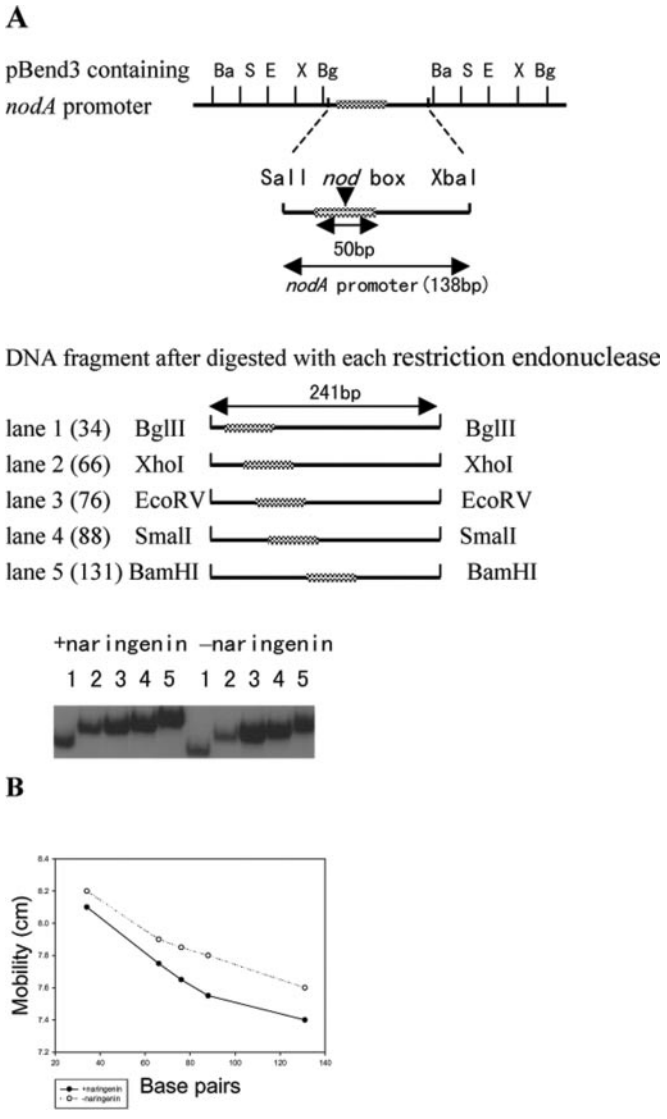

Figure 2. Determination of NodD-induced DNA bending on wild-type *nodA nod* box by circular permutation assay. (**A**) A 138 bp DNA fragment containing the *nod* box of *nodA* was cloned into the plasmid pBend3. The resulting plasmid was digested with the restriction endonucleases BglII (lane 1), XhoI (lane 2), EcoRV (lane 3), SmaII (lane 4) or BamHI (lane 5). The resulting fragments were incubated with cell extracts from the *R.leguminosarum* strains 8401(pIJ1518) in the presence or absence of 40 μM naringenin and size fractionated at room temperature. The position of the *nod* box in pBend3 and in each fragment after digested with each restriction endonuclease mentioned above was drawn not to scale in the upper, with the nucleotides separating the middle of *nod* box from the left end of each DNA fragment included in the bracket. Bg, BglII (lane 1); X, XhoI (lane 2); E, EcoRV (lane 3); S, SmaII (lane 4); and Ba, BamHI (lane 5). (**B**) Graphical representation of NodD-induced DNA bending on the *nod* box. Migration (*y*-axis) was plotted as a function of the number of nucleotides separating the middle of *nod* box from the left end of each fragment.

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 pMP-2D, 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 transformational 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).

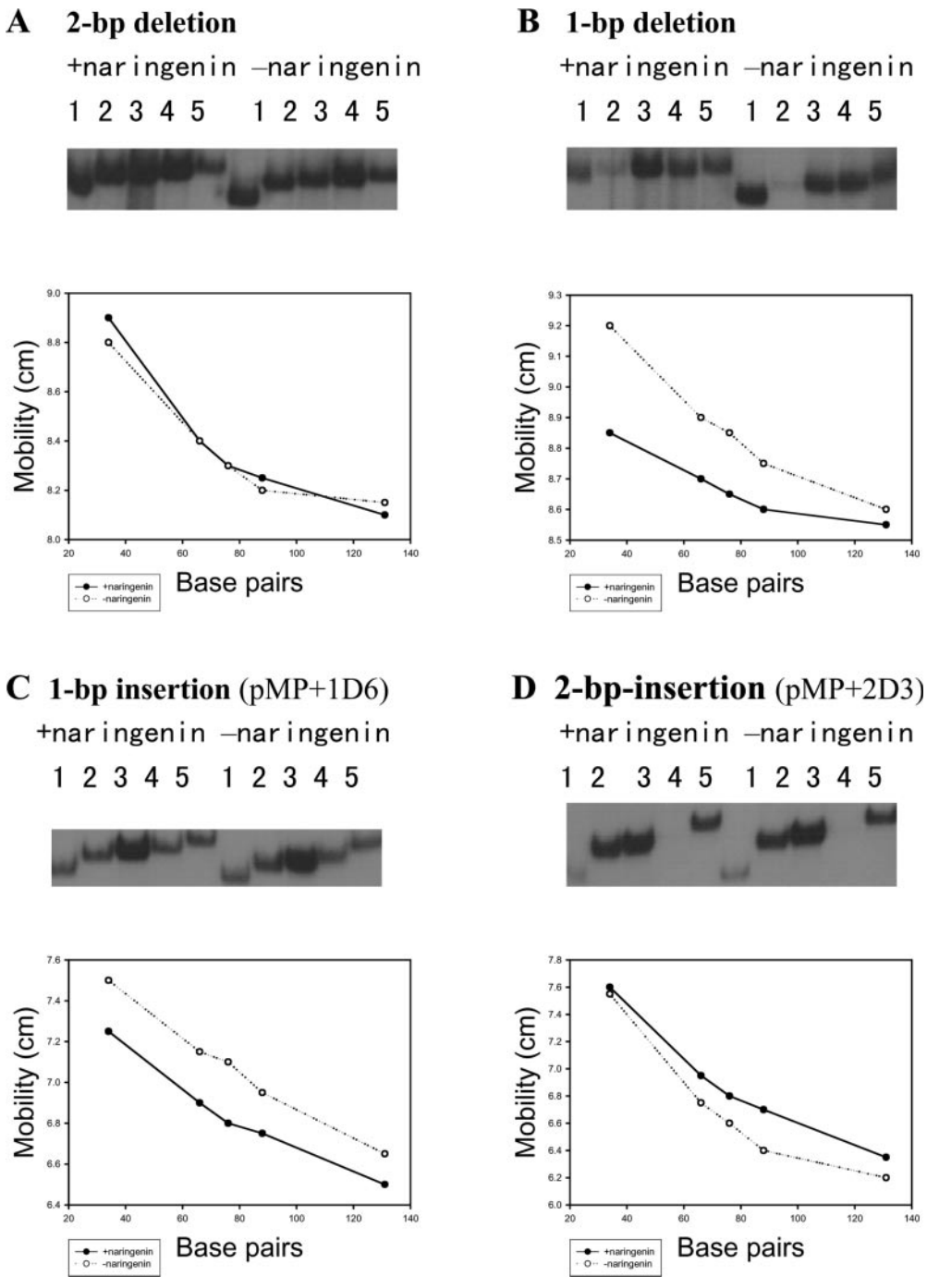

Figure 3. Determination of NodD-induced DNA bending on mutant *nodA nod* boxes by circular permutation assay. Four DNA fragments with lengths of 136, 137, 139 or 140 bp, containing *nod* box, were cloned into the plasmid pBend3. Each resulting plasmid was digested with the restriction endonucleases BglII (lane 1), XhoI (lane 2), EcoRV (lane 3), SmaI (lane 4) or BamHI (lane 5) [the upper part of (**A**–**D**)]. The resulting fragments were incubated with cell extracts from the *R.leguminosarum* strains 8401(pIJ1518) in the presence or absence of 40 μM naringenin and size fractionated at room temperature. The lower part of (A–D) is the graphical representation of NodD-induced DNA bending on *nodA nod* box mutants. Migration (*y*-axis) was plotted as a function of the number of nucleotides separating the middle of *nod* box from the left end of each fragment. The position of the *nod* box of each fragment was similar to that shown in Figure 2. The band in track 4 in (D) is weak but could be detectable in the X-film when subjected to autoradiography for a longer time, and the mobility values for this fragment plotted on the graph were from the X-film.

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

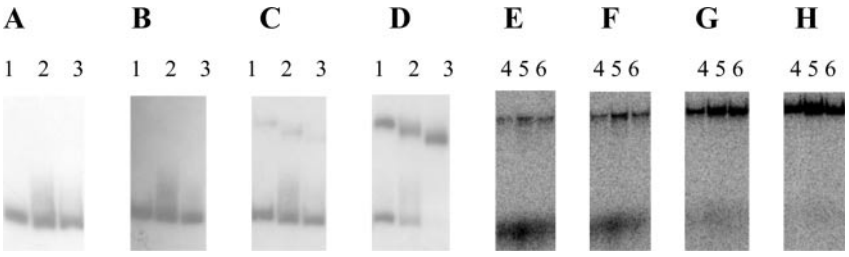

Figure 4. The binding affinity of NodD to wild-type *nodA* promoter or its mutant derivatives. (**A–D**) The cell extracts from the *R.leguminosarum* strain 8401(pIJ1518) incubated in the absence of naringenin used in (D) is 10-fold greater than that in (C) and 10-fold greater than that in (B) and 10-fold greater than that in (A). The fragments used in (A–D) lanes 1, 2 and 3 were from *nodA* promoter of 2 bp deletion, 1 bp deletion mutants and the wild-type control, respectively. (**E–H**) 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). The fragments used in (E–H) lanes 4, 5 and 6 were from *nodA* promoter of 2 bp insertion, 1 bp insertion mutants and the wild-type control, respectively.

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 −naringenin	Units β-galactosidase 8401(pKT230)	8401(pIJ1518) −naringenin	+naringenin
nodA promoter derivatives						
I: Wild type	pMPD	44	48	83	85	2681
II: 2 bp deletion	pMP-2D1	44	49	93	372	746
III: 1 bp deletion	pMP-1D	42	30	116	154	138
IV: 1 bp insertion	pMP+1D6	55	53	2443	1601	2286
V: 2 bp insertion	pMP+2D3	70	67	2319	2667	1942
NodD-mediated repression of itself						
I: Wild type	PMP220D			371	131	113
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.
ND, Not determined.

underlying LTTR-induced transcription of its target gene relates to protein-directed DNA bends (22,26).

In this work, our results showed that naringenin sharpened a NodD-induced DNA bend on the wild-type *nodA nod* box, which correlated with *nodA* transcription activation and suggested that the transcription induction trigger of inducible *nod* genes is correlated with changes on NodD-induced DNA bend on the *nod* box (Figure 2 and Table 3). Further studies showed that when the distance between the two half-sites of the *nod* box of *nodA* was slightly shorter than that of wild type, as in the case of 1 bp deletion, NodD induced relaxed DNA bend on *nodA* promoter in the presence and absence of naringenin, the transcription of *nodA* was similar to or slightly higher than that of wild type in absence of naringenin and lower than the wild type in the presence of naringenin. When the distance between the two half-sites was slightly longer than that of wild type, as in the two striking cases in pMP+1D6 and pMP+2D3, NodD introduced sharpened DNA bending at the *nodA* promoter, and *nodA* transcription was initiated constitutively with no requirement for the inducer naringenin (Figure 3 and Table 3). Although such distance modulation between the two half-sites of *nod* box changed the relative binding affinity of NodD to the *nod* box (Figure 4), there was no obvious relationship between the severe transcription changes of *nodA* promoter and that of binding affinity. These results indicate strongly that it was the artificial modulation on DNA bend that severely affected NodD-mediated transcriptional control.

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. It is interesting that in the two striking cases as in pMP+1D6 and pMP+2D3, *nodA* transcription is also initiated in a NodD-independent manner, which suggests there may be an additional regulator besides NodD, which may be a repressor, involved in the regulation of inducible *nod* gene. It is possible that the role of the NodD-induced DNA bend is to relieve the repression of such repressor upon inducible *nod* gene transcription by inhibiting the binding of the potential regulators to the promoter. Figure 5 presents a model summarizing these findings. The NodD tetramer anchors the two half-sites of the promoter of inducible *nod* gene in the absence or presence of inducer and causes a bend. The NodD-induced DNA bend, perhaps due to the repression of a suspicious repressor, would not allow RNA polymerase to form active transcriptional open complex in the absence of inducer. NodD in response to the inducer naringenin sharpens the bend, which may help to relieve the repression of such repressor and allow RNA

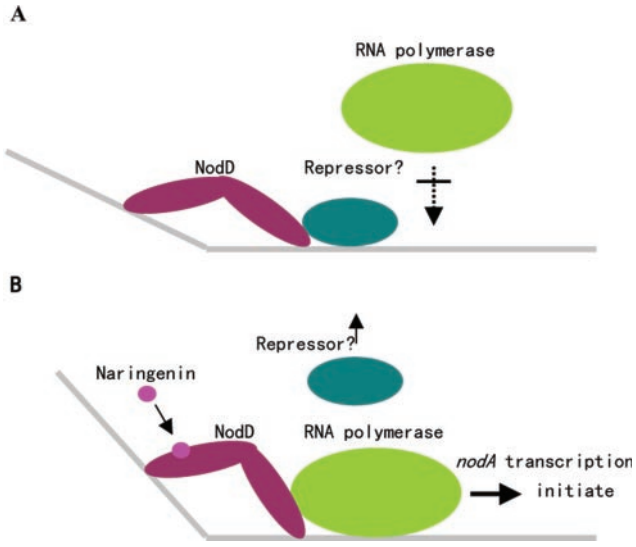

Figure 5. Model of NodD-mediated transcriptional induction. The NodD tetramer anchors the two half-sites of the promoter of inducible *nod* gene in the absence or presence of inducer and causes a bend. The NodD-induced DNA bend, perhaps owing to the repression of a suspicious repressor, would not allow RNA polymerase to form active transcriptional open complex in the absence of inducer. NodD in response to the inducer naringenin sharpens the bend, which may help to relieve the repression of such repressor and allow RNA polymerase to form active transcriptional open complex upon the inducible *nod* gene. In this figure, we employ two connected ellipses to represent the NodD tetramer and a broken line to represent the DNA duplex of the promoter of inducible *nod* gene. The suspicious repressor and its binding site on the promoter of inducible *nod* gene need to be determined.

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 $\mu_m/\mu_e = \cos(\alpha/2)$, the angle is determined by the ratio between μ_m and μ_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 μ_m/μ_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, effecting 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 groELc 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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