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Research Note

Characterization of the Overlapping Promoters of *nolB* and *nolW,* Two Soybean Cultivar Specificity Genes from *Rhizobium fredii* Strain USDA257

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The transcripts of *nolW* and *nolB,* two divergently oriented cultivar specificity genes of *Rhizobium fredii* strain USDA257, are known to be initiated 14 bp apart from promoters that face one another. We show here that expression of *nolB* is dependent both on induction with flavonoid signals and on the regulatory gene, *nodD1.* Expression of *nolW* is constitutive and independent of flavonoids and *nodD1.* Normal expression of *nolB* is retained with a promoter that extends only 61 bp upstream of the transcript start site, but it is lost if an additional 24 bp are removed. Substantial expression of *nolW* is retained with a promoter that contains only 34 bp of DNA upstream from the transcript initiation site. The dual control region for the two genes is thus only about 109 bp in length.

Cultivar-specific nodulation of soybean (*Glycine max* (L.) Merr.) by *Rhizobium fredii* strain USDA257 is controlled by a locus termed *nolXWBTUV* (Heron et al. 1989; Meinhardt et al. 1993). When all six of these genes are intact, nodulation of improved cultivars such as McCall is blocked at the stage of infection (Chatterjee et al. 1990). If any one of them is inactivated nodulation is allowed to proceed normally and nitrogen is fixed. *nolXWBTUV* is not known to influence the symbiotic interaction between USDA257 and most other legume hosts (our unpublished data), but it does exert a negative effect on symbiosis with several species of *Erythrina,* a woody legume that is only distantly related to soybean (Krishnan and Pueppke 1994). A sym plasmid locus, *nolXWBTUV* is divided into three transcriptional units (Kovács et al. 1995). *nolB, nolT, nolU,* and *nolV* are closely spaced and comprise a single unit. *nolW* lies upstream of *nolB* and is oriented divergently from it. *nolX* is of the same polarity as *nolW* and lies 281 bp downstream from it (Fig. 1). We have defined each of the three transcript initiation sites and have sequenced all of the open reading frames (Meinhardt et al. 1993; Kovács et al. 1995).

Expression of most nodulation (*nod*) genes is under the control of one or more copies of the regulatory gene, *nodD,* and stimulated by flavonoids from the plant host (Schlaman et al. 1992; Pueppke 1996). Analysis of gene fusions has confirmed that expression of *nolX* and *nolBTUV,* but not *nolW,* is greatly enhanced by several of these signal compounds (Meinhardt et al. 1993; Bellato et al. 1996). This pattern of flavonoid responsiveness, which has been verified independently by RNA dot blots and transcript analysis (Kovács et al. 1995), is unusual, because the *nolXWBTUV* locus lacks the consensus promoter sequences that are thought to be essential

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Table 1. Expression of *nolB* and *nolW* in *R. fredii* USDA191 and USDA191NodD1⁻

Strain	Gene	β-Galactosidase activity (Miller units)[a] Uninduced	Flavonoid-induced
USDA191(pMP220)	None	175 ± 38	164 ± 41
USDA191(pMP275.6)	*nolW*	1,854 ± 132	1,923 ± 75
USDA191(pMP275.1)	*nolB*	149 ± 8	1,800 ± 194
USDA191NodD1⁻(pMP220)	None	115 ± 16	99 ± 21
USDA191NodD1⁻ (pMP275.6)	*nolW*	1,821 ± 46	1,822 ± 81
USDA191NodD1⁻ (pMP275.1)	*nolB*	203 ± 55	201 ± 24

[a] Bacteria were grown for 7 h in the presence or absence of 2 μM apigenin as inducer. Values represent means ± standard deviations from replicate experiments.

Table 2. Delimitation of the promoter regions of *nolB*[a]

Fusion	Promoter terminus[b]	β-Galactosidase Activity (Miller units) 2 μM apigenin	Ethanol	Fold induction
pMP275.1	–176	1,882 ± 273	182 ± 85	10.3
pB2	–70	2,215 ± 78	179 ± 25	12.4
pB3	–61	2,053 ± 187	191 ± 17	10.7
pB4	–37	89 ± 25	70 ± 16	1.3
pB5	–25	153 ± 15	113 ± 7	1.4
pB6	–18	3,166 ± 142	2,967 ± 31	1.1
pB7	–15	4,622 ± 142	3,910 ± 392	1.2
pMP220	control	58 ± 4	58 ± 20	1.0

[a] Bacteria were grown for 7 h in the presence or absence of inducer. Values represent means ± standard deviations from replicate experiments.
[b] Distance in base pairs from the transciptional initiation site.

for mediating flavonoid inducibility (Schlaman et al. 1992). Here we focus on defining the divergent promoter region that lies between *nolB* and *nolW*. This short 155-bp block of bases (Fig. 2) contains the upstream terminus of the flavonoid-responsive *nolBTUV* transcript and that of the flavonoid-nonresponsive *nolW* transcript (Kovács et al. 1995). The transcript start sites are just 14 bp apart and oriented toward one another in a face-to-face arrangement of the type described by Beck and Warren (1988).

The role of *nodD* in expression of *nolB* and *nolW* was assessed with two plasmids, both based on promoter probe pMP220 (Spaink et al. 1987). Each contained a 257-bp PCR-amplified fragment that spans the nontranslated region between the two genes and includes 51 bp of the 5′-coding region of each (Kovács et al. 1995). *nolB* is oriented toward *lacZ* of the vector in pMP275.1, and *nolW* assumes this orientation in pMP275.6. Since *nodD*-negative mutants of strain USDA257 are not available, we transferred the plasmids to *R. fredii* USDA191 and to mutant USDA191NodD1⁻, which contains a kanamycin-resistance cassette in the *Bam*HI site of *nodD1* (Appelbaum et al. 1988). The sequences of *nodD1* and its promoter region are identical in the two strains

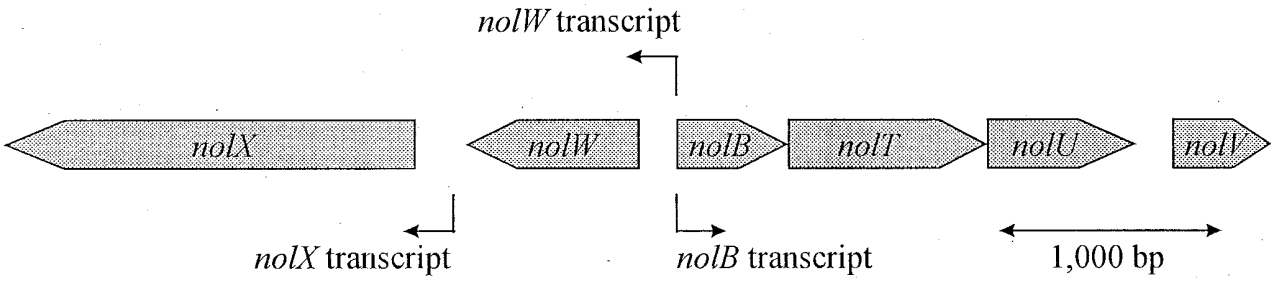

Fig. 1. Map of the cultivar-specificity region *nolXWBTUV* of *Rhizobium fredii* strain USDA257.

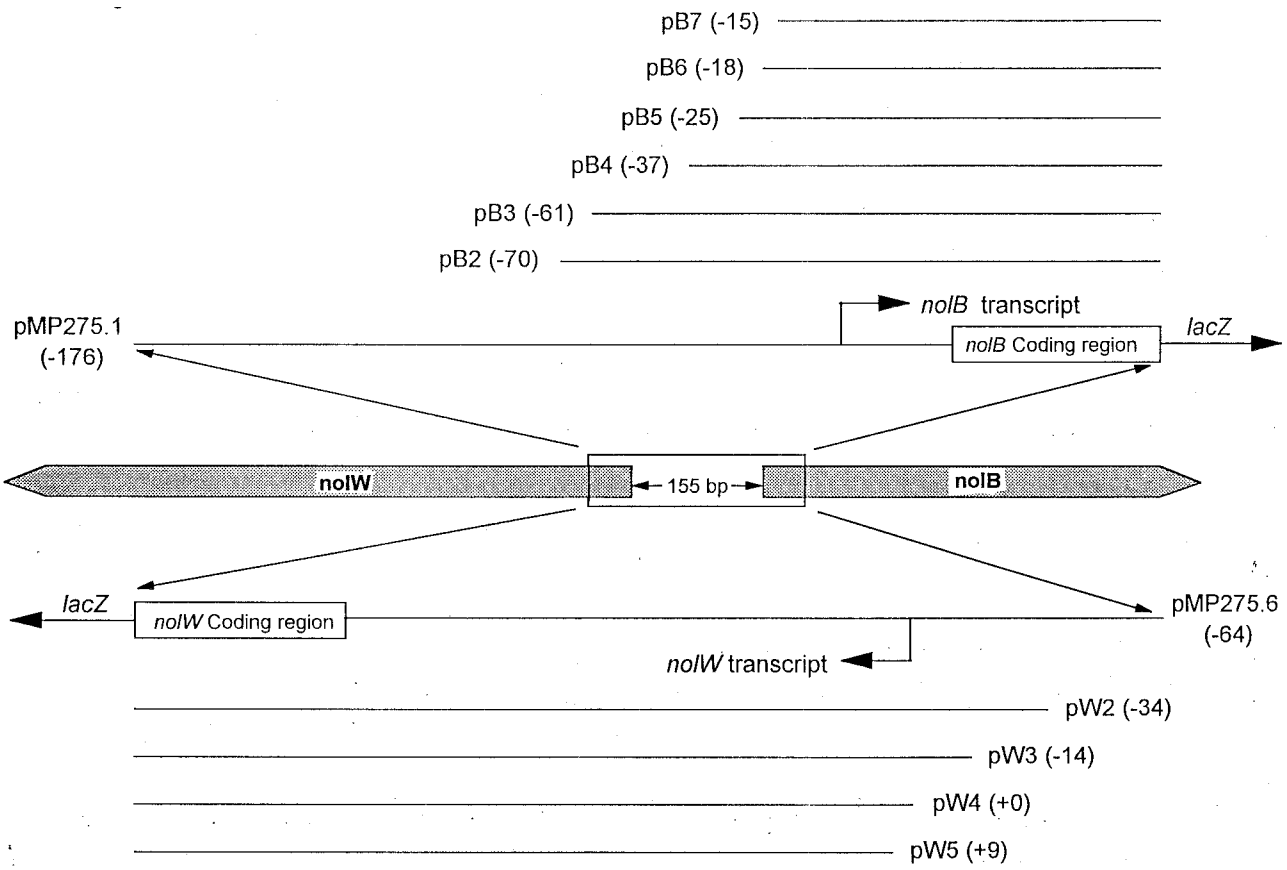

Fig. 2. The divergent *nolW/nolB* promoter region of *Rhizobium fredii* strain USDA257. The two open reading frames are represented by shaded arrows pointing in opposite directions, and the PCR-amplified promoter region is boxed. It includes the first 51 bp of *nolW,* a block of 155 bp that separates the two genes, and the first 51 bp of *nolB.* pMP275.1 and the *nolB* deletion derivatives prepared from it appear in exploded form above the shaded arrows. pMP275.6 and the *nolW* deletion derivatives prepared from it appear in exploded form below the shaded arrows. The number in parentheses that follows the name of each deletion indicates the position of the distal terminus of the remaining promoter with respect to the transcript start site.

(Appelbaum et al. 1988; Krishnan et al. 1995). Cells were induced with the flavone apigenin and β-galactosidase activity measured as described (Krishnan and Pueppke 1991; Bellato et al. 1996).

The *nolW* promoter increased β-galactosidase activity to more than 10 times the background level, whether or not the cells had been induced with apigenin. This activity was independent of *nodD1* (Table 1). In contrast, the *nolB* promoter elevated β-galactosidase activity about 12-fold, but only if *nodD1* was intact and the cells had been exposed to the flavonoid. These observations are significant for several reasons. First, they establish that expression of *nolW* resembles that of regulatory nodulation genes, including *nodD* itself and *syrM* (Györgypal et al 1991; Schlaman et al. 1992; Pueppke 1996), as well as *nodW* (Loh and G. Stacey, unpublished observations). In this sense, *nolW* differs fundamentally from the majority of *nod* genes. Second, we show that expression of *nolB*, like that of *nolJ* of *R. fredii* strain USDA201 (Boundy-Mills et al. 1994), depends not only on host signals, but also on the *nodD* regulatory circuit. Third, our data confirm that *nodD2,* a second *nodD* copy that is present and active in USDA191NodD1^{-} (Appelbaum et al. 1988), cannot sustain expression of *nolB*. This finding is in accord with the assignment of only a minor symbiotic role to this gene (Appelbaum et al. 1988).

We next employed deletion analysis to precisely define the promoters of *nolB* and *nolW*. The DNA insert from pMP275.6 was excised as a *Xba*I/*Hin*dIII fragment and ligated into pGEM7Zf(+), a multicopy vector from Promega Biotech. This plasmid was linearized with *Bgl*II, filled in with α-phosphoorothioate deoxynucleotide triphosphates (according to the protocol of Promega Corp.), and then digested with *Cla*I to create a site for deletion into the *nolB* promoter. Following partial digestion with exonuclease III and religation of the deleted derivatives, inserts were cloned into *Bgl*II/*Eco*RI-treated pMP220. A similar protocol was utilized to create progressive deletions into the *nolW* promoter. In this case, the orientation of the insertion in pGEM7Zf(+) was reversed; the plasmid was linearized with *Hin*dIII and cut with *Sal*I prior to digestion with exonuclease III. Each deletion endpoint was established by nucleic acid sequencing, and the promoter activities of the pMP220 derivatives were assessed in the USDA257 background as described.

Figure 1 positions of the dual promoter region with respect to the divergent open reading frames of *nolW* and *nolB.* pMP275.6, which has *nolW* oriented toward *lacZ*, increased β-galactosidase levels by about 15-fold compared to that of control plasmid pMP220 (906 to 1,071 Miller units for pMP275.6 versus 60 to 66 Miller units for the control). Deletion to positions –34 and –14 with respect to the transcriptional start site of the gene (Fig. 2) reduced expression to 7.3- and 6.3-fold, respectively, of control levels, indicating that significant activity is retained with a promoter that is only 34 bp in length. Removal of the entire promoter by deletion to positions 0 and +9, however, effectively abolished expression of the gene (1.2- to 1.7-fold induction as compared to the control plasmid). Expression of *nolW* by all of these derivatives was constitutive.

Table 2 gives the results of analogous experiments with the *nolB* promoter. Deletion to position –61 with respect to the transcriptional start site had no significant effect on expression of the gene, but removal of an additional 24 bp to create pB4 abolished promoter activity. Further deletion to position –18 or –15 caused β-galactosidase activity to reappear at a constitutive level that was about twice that supported by pMP275.1 (Table 2). We attribute this exaggerated activity to an artifactual promoter created during the deletion and ligation process (see Hunt et al. 1994, for example).

In summary, our deletion analysis confirms that only about 109 bp of DNA are required for full activity of *nolB* and substantial activity of *nolW.* From left to right (Fig. 2), this includes 61 bp upstream of the *nolB* transcript, the 14 bp that lie between the two transcripts, and 34 bp upstream of the *nolW* transcript. Approximately 25 bp of DNA usually separate the transcript start sites of conventional *nodD*-dependent genes from the proximal termini of their *nod* box promoters (Fisher et al. 1987, 1988; deMaagd et al. 1989; Spaink et al. 1989; Wang and Stacey 1991; Goethals et al. 1992; Göttfert 1993). Expression of *nodA* from *R. leguminosarum* bv. *viciae* is attenuated if sequences between the *nod* box and the transcript are deleted, but removal of sequences 5′ to the *nod* box has no effect on activity (Spaink et al. 1987). The minimum promoter that is necessary for wild-type *nod* gene expression in *R. leguminosarum* bv. *viciae* consequently extends about 75 bp upstream from the beginning of the transcript.

Our deletion analysis confirms that the minimum promoter of *nolB* is nearly this long. We have examined this region in detail, searching for features that might explain its activity. Neither the classical *nod* box consensus sequence (Spaink et al. 1987), the T-N_{11}-A LysR promoter motifs (Goethals et al. 1992), nor the 9-bp consensus repeats of Wang and Stacey (1991) are identifiable in this area. There are no significant direct or indirect repeats. We have not been able to find meaningful sequence homology between the minimal *nolB* promoter and any other prokaryotic promoter regions. Although the precise *nolJ* and *nolX* promoters have not yet been defined, we have aligned the minimal *nolB* promoter with sequences 5′ to the coding regions of *nolJ* and *nolX* (Sadowsky et al. 1988; Kovács et al. 1995). There were no homologies, and so the striking functional similarities of the three promoters must be based on features other than sequence conservation.

Although the deduced amino acid sequence of NolB has no significant similarity to other known polypeptides, NolW (Meinhardt et al. 1993) is homologous to proteins that function as part of Type III secretion systems of gram-negative bacteria (Cornelis 1994; Salmond 1994). Some of these proteins, including HrpA of *Pseudomonas solanacearum* (Gough et al. 1992; VanGijsegem et al. 1995), HrpA1 of *Xanthomonas campestris* pv. *vesicatoria* (Fenselau et al. 1992; Wengelnik et al. 1996), and HrpH of *P. syringae* pv. *syringae* (Huang et al. 1992), are present in plant pathogens, where they are thought to function in the release of elicitors of the hypersensitive reaction (Salmond 1994). The genes that encode them are expressed under conditions of nutrient limitation and embedded within complex operons. Although *nolW* is part of a multigenic cultivar-specificity region, it is monocistronic and positioned such that its expression likely influences that of *nolB* and vice versa (Beck and Warren 1988). Now that the *nolB/nolW* control region has been defined, it should be possible to precisely sort out the interactions between these two important promoters.

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