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Interaction of a Rhizobial DNA-Binding Protein with the Promoter Region of a Plant Leghemoglobin Gene[1]

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A nucleotide sequence was identified approximately 650 bp upstream of the *Sesbania rostrata* leghemoglobin gene *Srglb3* start codon, which interacts specifically with a proteinaceous DNA-binding factor found in nodule extracts but not in extracts from leaves or roots. The binding site for this factor was delimited using footprinting techniques. The DNA-binding activity of this factor was found to be heat stable, dependent on divalent cations, and derived from the (infecting) *Azorhizobium caulinodans* bacteria or bacteroids (*A. caulinodans* bacterial binding factor 1, AcBBF1). A 9- to 10-kD protein was isolated from a free-living culture of *A. caulinodans* that co-purifies with the DNA-binding activity (*A. caulinodans* bacterial binding protein 1, AcBBP1) and interacts specifically with its target (*S. rostrata* bacterial binding site 1, SrBBS1). The amino acid sequence of the N-terminal 27 residues of AcBBP1 was determined and was found to share significant similarity (46% identity; 68% similarity) with a domain of the herpes simplex virus major DNA-binding protein infected cell protein 8 (ICP8). An insertion mutation in the SrBBS1 was found to result in a substantial reduction of the expression of a *Srglb3-gus* reporter gene fusion in nodules of transgenic *Lotus corniculatus* plants, suggesting a role for this element in *Srglb3* promoter activity. Based on these results, we propose that (a) bacterial *trans*-acting factor(s) may play a role in infected cell-specific expression of the symbiotically induced plant *lb* genes.

The induction of nitrogen-fixing root and stem nodules on leguminous plants by soil bacteria belonging to the Rhizobiaceae involves the fine-tuned interaction between the two symbiotic partners, including multiple (regulatory) signals that go back and forth between the bacterium and the plant to coordinate expression of gene sets in both partners (Long, 1989; Nap and Bisseling, 1990; de Bruijn and Downie, 1991; Verma, 1992). Plant genes specifically induced during the symbiotic interaction (nodulin genes; van Kammen, 1984) are commonly divided into two classes: early nodulins, which correlate with the infection process and structural aspects of nodule ontogeny, and late nodulins, which participate in various aspects of nodule functioning, such as nitrogen assimilation, oxygen transport, and carbon metabolism (Verma and Delauney, 1988; Nap and Bisseling, 1990; Vance and Heichel, 1991).

Nodulin gene expression depends both on the presence of direct or indirect bacterial signals and on tissue- or cell-specific receptiveness in the plant. The regulation of expression of the early and late nodulin genes appears to differ substantially, and the exact nature of the rhizobial "symbiotic signals" and their targets, as well as the molecular basis for the tissue-specific (cell-specific) expression of nodulin genes are only beginning to be understood (de Bruijn et al., 1990; Nap and Bisseling, 1990; Sanchez et al., 1991; de Bruijn and Schell, 1992).

One line of research, aimed at elucidating the signal transduction pathways involved in early- and late-nodulin gene induction, has focused on the analysis of *cis*-acting elements in the 5′ upstream region of these genes and on the *trans*-acting factors interacting with them (de Bruijn et al., 1990; Sanchez et al., 1991; de Bruijn and Schell, 1992). A functional analysis of the *Sesbania rostrata* leghemoglobin *Srglb3* gene promoter region in transgenic *Lotus corniculatus* and tobacco (*Nicotiana tabacum*) plants has revealed the presence of two positive regulatory regions and an ATG-proximal element, between coordinates −429 and −48 (relative to the ATG start codon of the gene), involved in nodule-specific (infected cell-specific) gene expression (de Bruijn et al., 1989; Szabados et al., 1990). A similar ATG-proximal element has been found in the soybean leghemoglobin *lbc3* and late-nodulin N23 promoter regions (Stougaard et al., 1990; de Bruijn and Schell, 1992). The apparently infected cell-specific expression pattern of the *lb* promoter in transgenic *Lotus* plants suggests

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Abbreviations: AcBBF, *A. caulinodans* bacterial binding factor; BBF, bacterial binding factor; BBP, bacterial binding protein; BS1 (2), binding site 1 (2); Gus, glucuronidase; HMG, high mobility group; HPS, heparin Sepharose; HSV, herpes simplex virus; ICP8, infected cell protein 8; Lb, leghemoglobin; NAT, nodule A/T; NLS, nuclear localization signal; NXB, extraction buffer containing 42 M NaCl; 1.2NXB, extraction buffer containing 1.2 M NaCl; NXBCa, NXB buffer containing 20 mM $CaCl_2$; RP-C4, reverse-phase C4; SrBBS, *S. rostrata* bacterial binding site.

that the physical presence of bacteroids in the plant cell cytoplasm may be essential for the induction of this late-nodulin gene (de Bruijn et al., 1990; de Bruijn and Schell, 1992).

Gel retardation (gel shift) assays have been used to characterize the interactions of DNA-binding proteins from different tissues with the 5′ upstream regions of soybean and *S. rostrata lb* genes. A nodule-specific *trans*-acting factor has been identified that binds two A/T-rich sequences in the 5′ upstream region of the soybean *lbc*3 gene (BS1 and BS2; Jensen et al., 1988) and the *S. rostrata glb*3 gene (BS2*; Metz et al., 1988). This factor has been designated as NAT2 (Jacobsen et al., 1990). NAT2 binding activity is barely detectable in roots and increases in root nodules until days 10 to 12 after rhizobial infection, the time when most late nodulins are induced (Jacobsen et al., 1990; Jensen, 1991).

The interaction between NAT2 and its binding sites is highly specific, because single bp transversions in BS1 of the *lbc*3 5′ upstream region result in drastic changes in binding affinity (Jacobsen et al., 1990). NAT2-like factors have also been found in nodule extracts from *Sesbania* and alfalfa (de Bruijn et al., 1988; Metz et al., 1988), as well as French bean (PNF-1; Forde et al., 1990), suggesting that they may represent universal *trans*-acting factors in legume nodules. The BS1, BS2, and BS2* DNA sequences also interact with distinct DNA-binding proteins from leaves and roots (de Bruijn et al., 1988; Metz et al., 1988; Jacobsen et al., 1990), including an HMG-I-like protein, that may be involved in chromatin scaffold attachment (Jacobsen et al., 1990; Jensen, 1991).

Here, we show that multiple functional BS1-/BS2-like elements are present in the 5′ upstream region of the *S. rostrata glb*3 gene, and we describe their interaction with *trans*-acting factors from different plant tissues. We also describe a distinct DNA element (SrBBS1) that specifically interacts with a *trans*-acting factor derived from the infecting *Azorhizobium caulinodans* bacteria and acts as a *cis*-acting element in transgenic *Lotus* plants. We report the isolation and characterization of an *A. caulinodans* AcBBP1, copurifying with the SrBBS1-binding activity, and we discuss the possibility that rhizobial *trans*-acting factor(s) may play a role in infected cell-specific *lb* gene expression in the nodule. Preliminary reports of these results were presented at the Eighth International Congress on Nitrogen Fixation in Knoxville, TN (Welters et al., 1990), the Lunteren Lectures on Molecular Biology (de Bruijn et al., 1989), and the Third International Congress of Plant Molecular Biology (Tuscon, AZ, October 1991).

MATERIALS AND METHODS

Biological Materials and Growth Conditions

Azorhizobium caulinodans ORS571 (Dreyfus et al., 1988) was grown at 37°C in TY medium (Beringer, 1974), supplemented with 500 mg L^{-1} of carbenicillin. *Rhizobium loti* NZP2037 (Hotter and Scott, 1991) and *Rhizobium meliloti* 1021 (Meade et al., 1982) were grown at 28°C in TY medium. *Sesbania rostrata* stem- and root-nodulation experiments were carried out as described by Pawlowski et al. (1987). *Lotus corniculatus* nodulation experiments were carried out as described by Szabados et al. (1990). Nodule and other plant tissues were frozen in liquid nitrogen after harvesting and stored at −70°C until use. *Escherichia coli* strain JM109 (Yanish-Perron et al., 1985) was used for plasmid transformation experiments and was grown at 37°C in LB medium (Miller, 1972). *Agrobacterium rhizogenes* strain AR10 (Hansen et al., 1989) was used for plant transformation experiments and was grown at 28°C in TY.

DNA Manipulation and Plasmid Transformation

Plasmid DNA preparation, restriction enzyme cleavage, and ligation experiments, as well as plasmid transformation experiments, were carried out as described by Maniatis et al. (1982).

Plant Transformation Experiments

Binary vectors were introduced into *A. rhizogenes* AR10 by conjugation, and the resulting transconjugants were used to transform *L. corniculatus* seedlings, as described by de Bruijn et al. (1990) and Szabados et al. (1990). Fully regenerated transgenic plants were nodulated, and the tissues were analyzed for Gus activity as described by Szabados et al. (1990).

Preparation of Nuclear Extracts

Nuclear extracts were prepared using a modification of the method described by Jensen et al. (1988). Extraction buffers (buffer C; Jensen et al., 1988), NXB and 1.2NXB were used sequentially. The enriched nuclei preparations were resuspended in the NXB buffer, sonicated, and incubated on ice for 30 min, and the lysate was clarified by centrifugation (15,000*g* for 15 min). The pellet was reextracted with the 1.2NXB buffer. Extracts were aliquoted, frozen in liquid nitrogen, and stored at −70°C. Extractions under binding conditions were carried out in NXBCa, followed by extraction with the 1.2NXB buffer in the absence of $CaCl_2$. The protein concentrations of the extracts were determined as described by Bradford (1976).

Bacterial Extracts

For the preparation of small-scale extracts, bacteria from a 100-mL saturated culture were pelleted and resuspended in 3 mL of NXB buffer. The cells were lysed using an Aminco French Press, by sonication, or by vortexing with one-third volume of $CHCl_3$. The $CHCl_3$ was evaporated in a hot water bath under the hood. The lysates were clarified by centrifugation (15,000*g* for 30 min at 4°C), and the supernatant was aliquoted and frozen or used directly for the gel shift assays. For the large-scale preparative protein isolation, a 10-L culture of *A. caulinodans* ORS571 was grown to saturation, and the cells were pelleted by centrifugation. The cells (about 30 g) were resuspended in 30 mL of NXBCa, lysed by French Press treatment, and centrifuged, and the pellet was sequentially extracted with NXB and 1.2NXB. The supernatants of the two extractions were combined.

Heat Treatment of the Extracts

Small volumes of extracts (up to 500 μL) were incubated in Eppendorf tubes in a water bath at the appropriate tem-

perature for 15 to 60 min. After incubation, the extracts were cooled in a ice bath and centrifuged, and the supernatant was used for gel shift assays. Larger volumes (>5 mL) were added dropwise to an equal volume of prewarmed NXB buffer.

HPS and Reverse-Phase Chromatography

After heat denaturation and clarification, the soluble proteins were dialyzed against HPS buffer (100 mMTris/HCl [pH 8], 100 mM NaCl), $CaCl_2$ was added to a final concentration of 20 mM, and the resulting solution was applied to an HPS column (30-mL volume, flow rate 2.5 mL min^{-1}) equilibrated with HPS buffer containing 20 mM $CaCl_2$. The column was washed with HPS buffer in the presence of 100 to 1000 mM NaCl gradient and 20 mM $CaCl_2$. The DNA-binding activity was eluted in HPS buffer containing 1000 mM NaCl buffer and lacking $CaCl_2$. The active fraction of the HPS column was applied to an RP-C4 column (Vydac RP-C4, 250 × 4.6 mm) after acidification with TFA. The separation of the proteins was achieved by using a programmed gradient of buffer A (0.05% TFA in H_2O) and buffer B (0.056% TFA in 80% acetonitrile). During the first 20 min, the column was washed with 100% buffer A; subsequently (up to the 40th min), the proportion of buffer A was linearly decreased to 65%; for the next 60 min, buffer A was proportionally decreased to 45%; and until the 110th min, it was decreased to 25%. The AcBBP1 protein was eluted after 59 min.

Renaturation of DNA-Binding Proteins

Proteins were separated by SDS-PAGE and visualized by Coomassie blue or silver staining. The stained bands were excised, and the proteins were eluted in elution buffer (50 mM Tris/HCl, 100 mM EDTA, 5 mM DTT, 150 mM NaCl, 0.1 mg mL^{-1} of BSA, and 0.1% SDS), denatured in denaturation buffer (6 M guanidinium/HCl, 100 mM KCl, and 0.1% Nonidet P-40), renatured in NXBCa buffer, and purified on a Sephadex G-25 column (to remove guanidinium chloride).

Protein and DNA Sequencing

N-terminal amino acid sequence analysis was conducted by using an Applied Biosystem (Foster City, CA) 477A pulsed liquid-phase sequenator equipped with an on-line phenylthiohydantoin amino acid analyzer (120A). Both the sequenator and analyzer were operated according to the manufacturer's specifications. The DNA sequence of the fragments used for the gel shift assays was determined by using an Applied Biosystems ABI 380B DNA sequenator.

Labeling of DNA Fragments for Footprinting and Gel Shift Analyses

The DNA fragments were excised from their vector plasmids (pUC18 or pUC19) with *Eco*RI/*Hin*dIII. The purified fragments were labeled with [^{32}P]dCTP by using the Klenow fragment of DNA polymerase for the footprint experiments and with [^{32}P]dATP or dCTP for gel shift assays, as described by Maniatis et al. (1982). The unincorporated nucleotides were separated using Nick columns from Pharmacia (Piscataway, NJ).

Gel Shift Assays

The gel shift assays were carried out essentially as described by Jensen et al. (1988). The basic binding buffer contained 10% glycerol, 25 mM Hepes (pH 7.9), 0.1 mM EDTA, and 1 mM DTT. As nonspecific competitor DNAs, pUC18, calf thymus, salmon sperm, poly(dIdC), and poly(dAdT) were used in concentrations ranging from 0.5 to 50 μg of DNA per reaction. pUC18 DNA was used in its supercoiled/linear form. Calf thymus and salmon sperm DNAs were sonicated to generate smaller fragments before use. The protein extracts were incubated with DNA in the appropriate binding buffer for 20 min at room temperature. The protein-DNA complexes were separated on native polyacrylamide gels (4–8%, 15 V cm^{-1}, 2–3 h, room temperature). The electrophoresis buffer consisted of 50 mM Tris, 50 mM boric acid, and 1 mM EDTA.

DNaseI and Exo III Footprinting Analyses

The equivalent of approximately 100,000 cpm of labeled DNA fragment was mixed with 5 μg of poly(dIdC), 1 to 30 μg of protein, and binding buffer, containing either 20 mM Mg^{2+} or 20 mMCa^{2+} cations, and incubated at room temperature for 20 min. After digestion for 2 min with 100 ng of DNase I, the reaction was stopped by the addition of 20 μL of 0.2 M EDTA (pH 8) and phenolization. After ethanol precipitation and resuspension in loading buffer, the samples were analyzed on a 9% sequencing gel. For localization of the protected region, an A > C cleavage reaction of the fragment was run simultaneously (Maniatis et al., 1982). For the Exo III reactions, the labeled DNA fragments were digested with an appropriate restriction enzyme to remove the label from one end. The equivalent of approximately 30,000 cpm of the remaining fragment was incubated with 5 μg of poly(dIdC) and 50 to 100 μg of protein in binding buffer containing 20 mM $CaCl_2$ for 20 min. The digestion was started by the addition of 100 to 250 units of Exo III, and the reaction was stopped after 15 to 30 min by the addition of 5 μL of 3 M sodium acetate and phenolization. The reaction products were analyzed as described above.

Northern and Western Blot Analysis

Northern blot analysis was carried out as described by Dehio and de Bruijn (1992). Western blot analysis was carried out as described by Felix (1989).

RESULTS

Search for BS1-/BS2-Like Motifs in the *S. rostrata glb3* 5′ Upstream Region

We previously reported the nucleotide sequence of the first 388 bp of the *Srglb3* 5′ upstream region, up to the *Eco*RI site (Metz et al., 1988; Welters et al., 1989; Fig. 1). To extend our analysis further upstream, we determined the sequence of the adjacent *Dra*I-*Eco*RI fragment (positions −777 to −387 relative to the *Srglb3* ATG in Fig. 1). Selected restriction sites in the *Srglb3* 5′ upstream region were deduced from the DNA sequence and used to subclone contiguous DNA fragments for gel shift assays (labeled 5′191, 5′20, etc.; Fig. 1). The

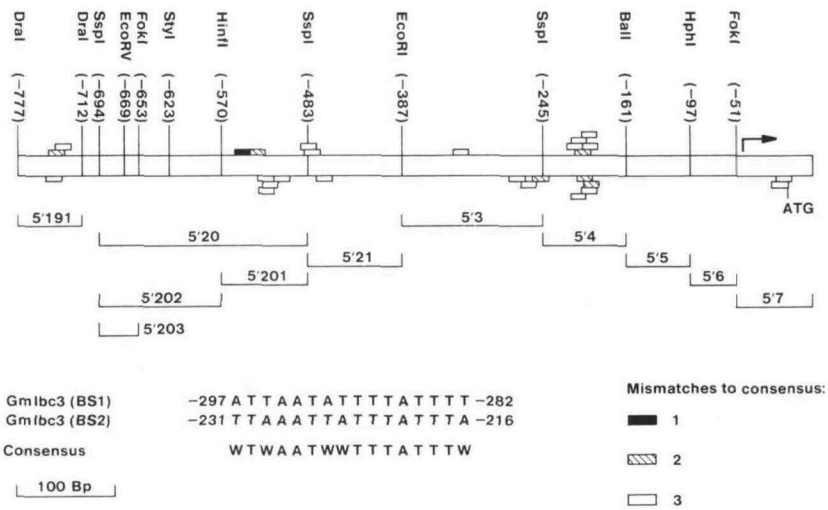

Figure 1. Mapping of BS2*-like sites in the *Srglb3* 5′ upstream region. The boxes indicate the degree of homology to the A/T consensus sequence derived from BS1 and BS2 of the soybean *lbc3* promoter (Jensen et al., 1988). Sequences with one mismatch to the WTWAATWWTTTATTTW (W = A/T) consensus sequence are denoted by solid boxes, two mismatches are indicated by striped boxes, and three mismatches are shown by open boxes. Homologies found on the coding strand are indicated above the line and on the noncoding strand beneath the line. The arrow indicates the start point of transcription (−48 relative to the ATG; Metz et al., 1988). The selected restriction sites are either important for the localization of binding activities or were used to subclone the *Srglb3* 5′ upstream region. Subfragments of the *Srglb3* 5′ upstream region are designated as 5′3–5′7 (Metz et al., 1988) and 5′191, 5′20, 5′21, 5′201–5′203 and were cloned into the *Sma*I site of vector pUC18.

Srglb3 5′ upstream region, up to position −777, was examined for the presence of DNA sequence motifs with similarity to BS1 and BS2 from the soybean *lbc3* promoter region (Jensen et al., 1988). For this purpose, a consensus sequence (WTWAATWWTTTATTTW, whereby W corresponds to A or T) was derived from BS1 (ATTAATATTTTATTTT) and BS2 (TTAAATTATTTATTTA) and DNA sequences with up to three mismatches with respect to the consensus sequence were identified using the FIND program from the University of Wisconsin Computer Group. The results are shown in Figure 1. A cluster of motifs with two or three mismatches was identified on fragment 5′4, corresponding to the BS2* site previously identified (Metz et al., 1988; de Bruijn et al., 1989). Another prominent cluster was found on fragment 5′20 (5′201). Other homologous motifs were found on fragments 5′191, 5′21, 5′3, and 5′7.

Fragments 5′191, 5′20, and 5′21 were radiolabeled, incubated in the presence of *S. rostrata* nodule, leaf, and root extracts and used for gel shift assays, as described by Metz et al. (1988). Fragments 5′191 and 5′21 formed distinct complexes with proteins from nodule and leaf extracts, but the binding efficiency was less than that found with fragment 5′4 (data not shown). Fragment 5′20 formed three complexes with nodule extract (Fig. 2, A–C). Competition experiments showed that complexes A and B, but not C, disappeared upon addition of increasing concentrations of unlabeled fragment 5′4 (carrying BS2*; Fig. 1) to the reaction mixture (Fig. 2). Both complexes A and C disappeared upon addition of unlabeled fragment 5′202 (Fig. 1) to the mixture (Fig. 2). These results suggest that fragment 5′20 interacts with (at least) two distinct factors, one of which is analogous to the factor interacting with fragment 5′4, previously identified by

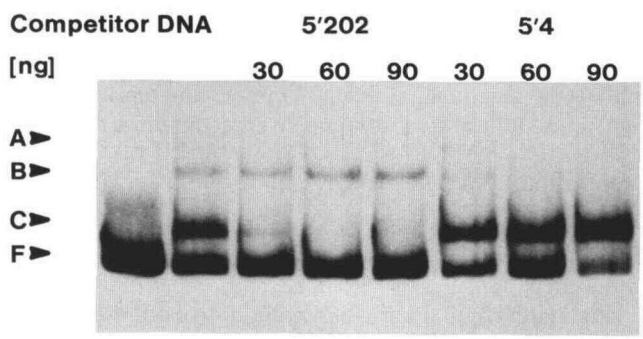

Figure 2. Interaction of fragment 5′20 with nodule extract and competition with fragments 5′202 and 5′4. Retardation gel of radiolabeled fragment 5′20 after incubation in the presence of stem-nodule extract and increasing amounts (indicated in ng) of unlabeled fragment 5′202 or 5′4 as specific competitor DNA. The free fragment without extract is shown in the first lane. The second lane shows complex formation in the presence of stem-nodule extract but absence of specific competitor DNA and three complexes are clearly visible (second lane; arrowheads A, B, and C). Increasing amounts of 5′202 DNA (30, 60, and 90 ng of DNA, respectively; lanes 3–5) inhibit the formation of complexes A and C, whereas the addition of 30, 60, and 90 ng of 5′4 DNA reduces the formation of complexes A and B but not C (lanes 6–8). Arrowhead F denotes the position of the free 5′20 fragment.

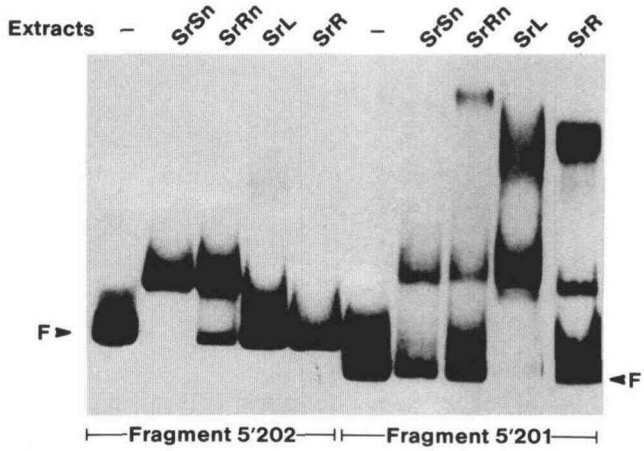

Figure 3. Interaction of fragments 5'202 and 5'201 with *S. rostrata* nodule, leaf, and root extracts. Retardation gel of radiolabeled fragments 5'202 and 5'201 after incubation in the presence of nuclear extracts from stem nodules (SrSn), root nodules (SrRn), leaves (SrL), and roots (SrR). Fragment 5'202 binds nodule-derived factors only, whereas fragment 5'201 shows a more general affinity for (distinct) factors from all tissues. Arrowhead F indicates the position of the free fragments.

Metz et al. (1988), and that the target sites for these factors are separated by the *Hin*fI site (position −570; Fig. 1). Moreover, the formation of the weak complex A, outcompeted by both fragments 5'4 and 5'202 DNA, is likely to be the result of both factors binding to the same target fragment.

To investigate these interactions further, the complex formation patterns of fragments 5'201 and 5'202 were compared. The results are shown in Figure 3. Fragment 5'201 formed distinct complexes with extracts from *S. rostrata* stem or root nodules, leaves, and roots, whereas fragment 5'202 only formed complexes with stem- and root-nodule extracts (Fig. 3). The complex pattern formation observed with fragment 5'201 was found to resemble the pattern observed with fragment 5'4 (Metz et al., 1988) and could be outcompeted by the addition of unlabeled fragment 5'4 DNA (data not shown). Complex formation with fragment 5'202 was clearly different (Fig. 3) and could not be outcompeted by the addition of fragment 5'4 DNA (data not shown). These differences were further accentuated by using other (nonspecific) competitor DNAs, such as salmon sperm, calf thymus, poly(dIdC), poly(dAdT), and pUC18 vector DNA. Complex formation of nodule extracts with fragment 5'201 could be abolished by the addition of 250-fold excess of poly(dAdT) but remained unaffected by the addition of the other nonspecific competitor DNAs (data not shown). Complex formation of nodule extracts with fragment 5'202 could not be abolished by the addition of up to 50,000-fold excess of the competitor DNAs listed above (data not shown).

Interaction between Fragment 5'202 and Nodule Extracts

Because fragment 5'202 interacted with a factor found exclusively in nodule extracts and appeared to lack A/T BS1-/BS2-type motifs, this factor-DNA interaction was investigated further. Formation of the complex was stimulated by Ca^{2+} (or Mg^{2+}) and was abolished by proteinase K (Fig. 4), suggesting that it is due to a cation-dependent protein-DNA interaction. Addition of increasing amounts of unlabeled fragment 5'202 resulted in a rapid disappearance of the complex (Fig. 4), suggesting that the protein-DNA interaction is specific. This was confirmed by the addition of a 1000-fold excess of unlabeled fragment 5'201 competitor DNA, which did not affect complex formation (Fig. 4).

Temperature Sensitivity of the DNA-Binding Factors

The temperature sensitivity of the DNA-binding protein(s) interacting with fragment 5'202 was also examined. Treating the (stem) nodule extracts at 50°C for 15 min resulted in total loss of DNA-binding activity, but extracts treated at 80 or 100°C for the same amount of time showed high DNA-binding activity (Fig. 5). Even incubation of the extract in boiling water for 60 min did not result in a loss of DNA-binding activity (see below). The protein-DNA interaction responsible for complex formation with fragment 5'201 showed a slightly different pattern of temperature sensitivity, in that it showed a high degree of heat stability at 100°C but was absent in extracts treated at 80°C (Fig. 5). Addition of proteinase inhibitors to the extracts before heat treatment abolished the differential temperature effects; complex formation could be observed with both fragments 5'201 and 5'202 at all four temperatures (data not shown). We infer that, in addition to the heat stability of the binding proteins, selective temperature sensitivities of specific proteases involved in the degradation of the DNA-binding proteins may influence the binding activity.

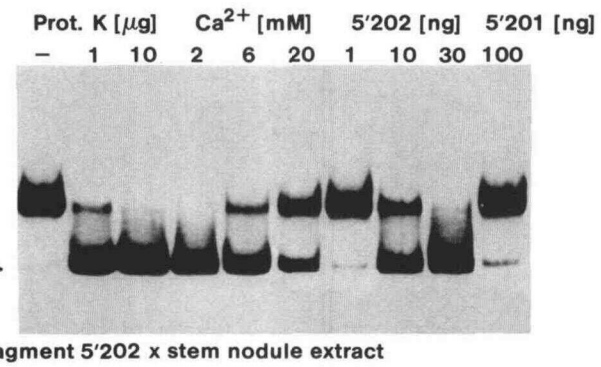

Figure 4. Properties of the nodule-derived binding factor interacting with fragment 5'202. Retardation gel of fragment 5'202 after incubation in the presence of stem-nodule extract under different conditions. Lanes 2 to 4 show the results of pretreating the extract for 15 min with different amounts (1 or 10 μg) of proteinase K (Prot. K). The first three lanes confirm the proteinaceous nature of the binding factor interacting with fragment 5'202. A 15-min treatment of the extracts with 0, 1, or 10 μg (indicated above the panel) of proteinase K at 37°C clearly abolishes binding activity to fragment 5'202 (lanes 1–3). Lanes 4 to 6 show that the complex formation is Ca^{2+} dependent, requiring >4 mM $CaCl_2$. Lanes 7 to 10 show that the complex formation is outcompeted by 30 ng of fragment (5'202) specific competitor DNA (lanes 7–9) but not by 100 ng of fragment 5'201 DNA (lane 10).

Factor Interacting with Fragment 5′202 Is Derived from the Infecting *A. caulinodans* Bacteria

Because it was difficult to isolate nodule nuclei that are completely free of bacteroids and bacteria for extract preparation, control binding experiments were carried out using extracts from cultures of the nodulating rhizobial strain *A. caulinodans*. Surprisingly, extracts prepared from free-living *A. caulinodans* cultures gave rise to the formation of a complex with fragment 5′202 (Fig. 6, lanes 3 and 4) that showed the same migration pattern as a complex found with stem-nodule extract (SrSn; Fig. 6, lane 2). Moreover, the binding activity from *A. caulinodans* cultures was also resistant to boiling (Fig. 6, lane 4; labeled 100°) and showed the same Ca^{2+} response as the nodule factor (data not shown). This factor was designated AcBBF1.

To determine whether the *A. caulinodans-S. rostrata* symbiotic system represented a unique case, extracts were also prepared from *L. corniculatus* nodules and from the *Lotus* symbiont *Rhizobium loti* and incubated with fragment 5′202. Complexes with a mobility similar to those observed with *S. rostrata* stem-nodule and *A. caulinodans* culture extracts were observed with both *L. corniculatus* nodule (LcN in Fig. 6, lane 5) and *R. loti* culture extracts (Rl in Fig. 6, lanes 6 and 7). The *R. loti* DNA-binding factor was also found to be resistant to boiling (Fig. 6, lane 7; 100°). Similar experiments were carried out using extracts from alfalfa or pea nodules and from the respective symbionts, revealing in each case a faint complex of a different mobility but similar migration pattern (data not shown).

Delimitation of the *Srglb3* SrBBS1

To delimit the binding site for the factor from *S. rostrata* nodules and free-living *A. caulinodans* or *R. loti* cultures

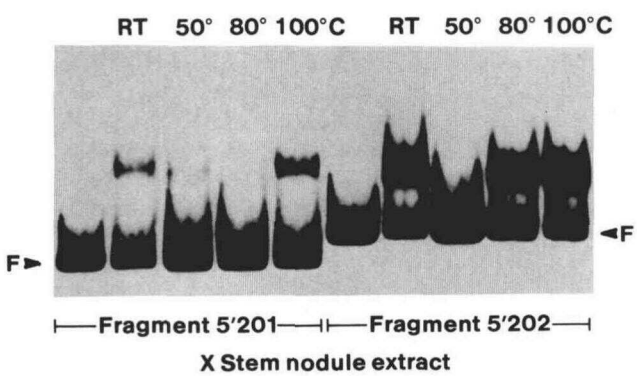

Figure 5. Heat stability of factors binding to fragments 5′201 and 5′202. Retardation gel of radiolabeled fragments 5′201 and 5′202 after incubation in the presence of stem-nodule extracts, pretreated for 15 min at room temperature (RT), 50, 80, and 100°C, respectively. In extracts without proteinase inhibitors, the binding factors interacting with fragments 5′201 and 5′202 exhibit different temperature stabilities. In stem-nodule extracts, the binding factor interacting with fragment 5′201 was totally degraded at 80°C, but it was quite stable at room temperature and 50 and 100°C. In contrast, fragment 5′202 could not form a complex with 50°C-treated extracts, but the binding factor was stable at room temperature, 80°C, and 100°C. Arrowhead F indicates the position of the free fragments.

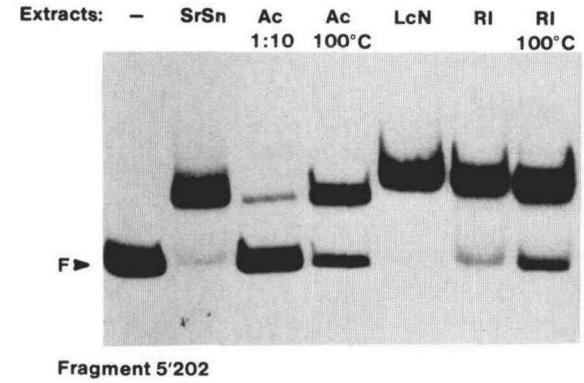

Figure 6. Interaction of fragment 5′202 with rhizobial extracts. Retardation gel of fragment 5′202 after incubation in the presence of extracts from *S. rostrata* stem-nodules (SrSn, lane 2), *A. caulinodans* (Ac, lanes 3 and 4), *L. corniculatus* nodules (LcN, lane 5), and *R. loti* (Rl, lanes 6 and 7). The 1:10 designation in lane 3 indicates a 1:10 dilution of the extract used. 100°C indicates that the extracts were pretreated at that temperature for 15 min. Arrowhead F indicates the position of the free 5′202 fragment.

(BBF1), fragment 5′202 was radiolabeled at the *Ssp*I terminus, digested with *Sty*I, *Fok*I, and *Eco*RV (Fig. 1), and incubated with *S. rostrata* stem-nodule extract. Complex formation was observed with the *Ssp*I-*Sty*I and *Ssp*I-*Fok*I fragments but not with the *Ssp*I-*Eco*RV fragment (data not shown). When fragment 5′202 was radiolabeled at the *Hin*fI terminus and digested with the same three enzymes, none of the resulting subfragments were found to give rise to complex formation with stem-nodule extracts (data not shown). These results suggest that the DNA sequences responsible for binding to BBF1 are contained within the *Ssp*I-*Fok*I fragment (5′203; Fig. 1).

Subsequently, fragment 5′202 was radiolabeled at either terminus, incubated with stem-nodule extract, and subjected to Exo III digestion (see "Materials and Methods"). Analysis of the resulting fragments on an acrylamide gel revealed that the region protected by the DNA-binding factor was located between positions −690 and −653 (data not shown). Fragment 5′202 was also subjected to DNase I digestion, after incubation with stem-nodule extract, revealing a protected region between positions −690 and −666 (Fig. 7A; data not shown). This analysis was repeated with a multimer of fragment 5′203. Six copies of fragment 5′203 were ligated in tandem to generate fragment 5′2036, and its structure was verified by DNA sequencing (data not shown). DNase I-footprinting analysis of fragment 5′2036, after incubation with stem-nodule extract, revealed the same protected region as found using fragment 5′202 (positions −690 to −666; Fig. 7). This region was designated SrBBS1. DNase I-hypersensitive sites were detected at positions −682 and −672 on the coding strand and at positions −673 and −684 on the noncoding strand (Fig. 7B). The same DNase I protection patterns were observed with extracts from stem nodules and *A. caulinodans* or *R. loti* cultures (Fig. 7A), suggesting that the DNA-binding factor is similar or the same in the different extracts.

The DNA sequence of SrBBS1 was found to be highly conserved at an analogous position (−734 to −710; P. Wel-

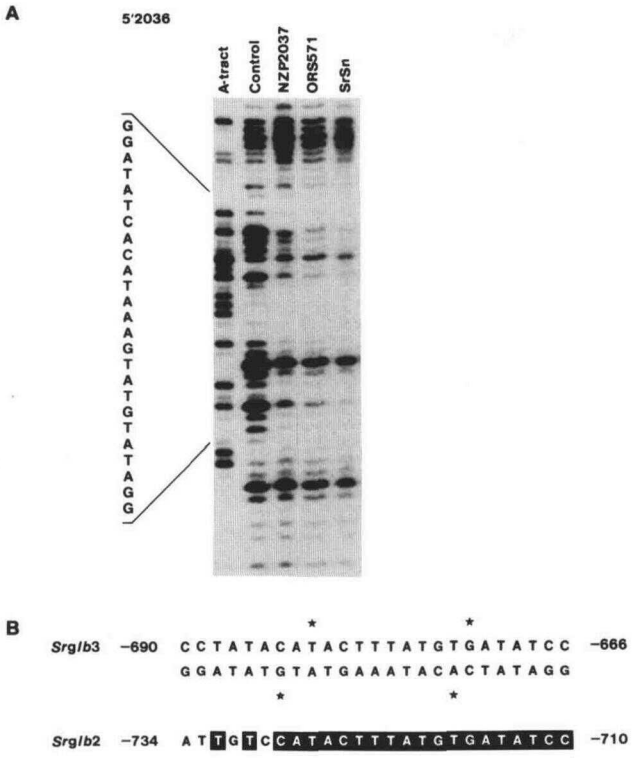

Figure 7. Delimitation of fragment 5'202 sequences responsible for interaction with AcBBF1. A, DNase I-footprinting gel of fragment 5'2036 after incubation in the absence of extract (lane 2, Control), *R. loti* 2037 culture extract (lane 3, NZP2037), *A. caulinodans* ORS571 culture extract (lane 4, ORS571), and *S. rostrata* stem-nodule extract (lane 5, SrSn). The samples were run on a 9% acrylamide DNA-sequencing gel, next to a A > C cleavage reaction of the same fragment (lane 1, A-tract). The DNA sequence of the protected region is shown on the left (−690 to −666). B (top), DNA sequence of the protected region from the *S. rostrata glb3* gene (−690 to −666). The stars indicate hypersensitive sites on both strands, and the results are based on several footprinting experiments with fragments 5'202 (both strands) and fragment 5'2036 (data not shown). B (bottom), DNA sequence of the −734 to −710 segment of the *S. rostrata Srglb2* 5' upstream region. The nucleotides homologous to the SrBBS1 are highlighted in black.

ters, B. Metz, and F.J. de Bruijn, unpublished data) in the *S. rostrata glb2* 5' upstream region (Fig. 7B).

Developmental Appearance of AcBBF1

S. rostrata stem nodules are induced synchronously at preformed infection sites (adventitious root sites) on the stem (de Bruijn, 1989). Therefore, this symbiotic system is particularly useful for the analysis of temporal patterns of gene expression and associated processes. To determine the temporal relationship between *S. rostrata lb* gene expression (Lb production) and the formation of the SrBBS1-AcBBF1 complex, 6-week-old *S. rostrata* plants were inoculated on the stem with *A. caulinodans* bacteria, and (infected) peels of the stem with adventitious root sites (and developing nodules) were harvested at different intervals after infection. Protein and RNA were isolated from the harvested material, and extracts were prepared for gel shift studies. Western blots containing total protein were probed with an anti-Lb antibody preparation, and northern blots carrying total RNA were probed with an *Srglb3* internal gene probe.

The western blot analysis revealed that Lb proteins could be detected first at d 12 after infection and steadily accumulated up to 7 weeks after infection, as reported previously (de Bruijn et al., 1989), and the 12-d time point coincided with the appearance of *lb* mRNA on northern blots (Felix, 1989). At d 0, a faint complex between fragment 5'202 (carrying SrBBS1) and stem extracts could already be observed, but the migration pattern of this complex was different from that observed with AcBBF1 from mature nodule extracts or bacterial cultures (Fig. 8). At d 12 after infection, a second complex comigrating with the 5'202-AcBBF1 complex could be observed, and shortly afterward the slower migrating first complex disappeared (Fig. 8). The second complex increased in intensity up to 7 weeks after infection (Fig. 8). Thus, there was a clear correlation between the appearance of *lb* mRNA and Lb protein and the appearance of the SrBBS1-AcBBF1 complex.

Purification of AcBBP1 from an *A. caulinodans* Culture

To isolate the protein(s) responsible for the SrBBS1-AcBBF1 complex formation, a 10-L culture of *A. caulinodans* bacteria was prepared. The bacterial cells were lysed in NXBCa, and

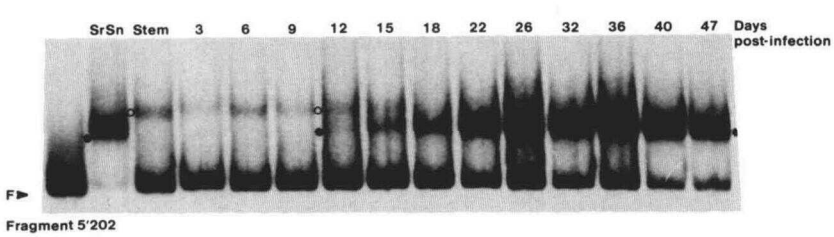

Figure 8. Developmental appearance of AcBBF1. Retardation gel of fragment 5'202 after incubation in the presence of *S. rostrata* stem and stem-nodule extracts from tissues harvested at different times (d) after infection (days post-infection) with *A. caulinodans* ORS571. The complex formed with mature (8 weeks postinfection) stem-nodule extract (SrSn, lane 2) and with extracts from nodule tissues starting 12 d postinfection is indicated by a solid circle. The complex formed with extracts from uninfected stems (Stem, lane 3) and infected stems up to 12 d postinfection is indicated by an open circle. Arrowhead F denotes the free 5'202 fragment.

the resulting lysate was clarified by centrifugation. The pellet was sequentially extracted with NXB and 1.2NXB, and the two soluble fractions were assayed for binding activity by using fragment 5'2036 as target DNA. Both fractions contained significant binding activity and were pooled for further purification. The pooled fractions were incubated in a boiling water bath for 30 min, and the denatured proteins and debris were removed by centrifugation. The supernatant was dialyzed and loaded on an HPS column. The column was washed with a 100 mM to 1 M NaCl gradient in the presence of Ca^{2+}, and subsequently proteins were eluted in the presence of 1 M NaCl and the absence of Ca^{2+}.

Three fractions (54–56) with a high degree of binding activity were identified (Fig. 9A). An aliquot of fraction 55 was loaded on an SDS-polyacrylamide gel and shown to contain three distinct proteins of 9 to 12 kD (Fig. 9B, lane HPS 55). The binding activity of the three different proteins was examined by excising the bands from the gel, eluting and renaturing the proteins, and carrying out gel shift experiments. Only the smallest protein was found to have a high binding activity to fragment 5'2036 (Fig. 9C) and was designated AcBBP1. The proteins of HPS fraction 55 were separated using an RP-C4 column, and the fractions were analyzed by SDS-PAGE (Fig. 9B, lanes 5–9). AcBBP1 was found in fraction 8 (Fig. 9B).

Determination of the N-Terminal Amino Acid Sequence of AcBBP1

The N-terminal amino acid sequence of AcBBP1 (27 residues) was determined (Fig. 10A). Analysis of the sequence revealed that most of the basic and hydrophobic amino acids were found in the first 15 residues, whereas the last 12 residues included six amino acids with polar chains and three with acidic side chains (Fig. 10A). The N-terminal amino acid sequence of AcBBP1 was compared with protein sequences in the SwissProt data bank. The highest degree of similarity (46% identity, 68% similarity over 23 amino acids) was found

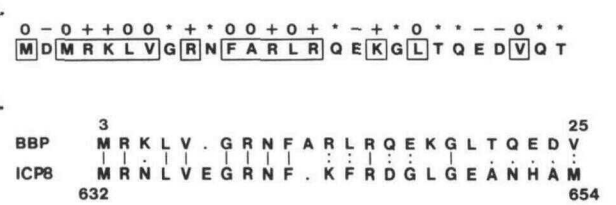

Figure 10. N-terminal amino acid sequence of AcBBP1. A, The sequence of the N-terminal 27 amino acids of AcBBP1, determined by N-terminal protein sequencing, is shown. The first 15 amino acids consist mainly of hydrophobic and basic amino acids; the 12 following residues have a more acidic and polar character. The hydrophobic basic residues are boxed. O denotes hydrophobic, * means polar, − means acidic, and + indicates basic amino acid residues. B, Amino acid sequence comparison of the N-terminal portion of AcBBP1 with a domain of the herpes simplex major DNA-binding protein ICP8 (Gao et al., 1988). Identical amino acids are denoted by vertical lines and similar amino acids are marked by dots.

with the major DNA-binding protein ICP8 of HSV (Gao et al., 1988; Fig. 10B). Other similarities that were found include an eight-amino acid match with a segment of the SUP1 suppressor protein from *Saccharomyces cerevisiae* (Breining and Piepersberg, 1986) and similarity of the hydrophobic/basic domain of AcBBP1 to the N-terminal domain of the DPR1 protein of *S. cerevisiae*, involved in interaction with the plasma membrane (Goodman et al., 1988; data not shown).

In Vivo and in Vitro Significance of the Binding Site for AcBBP1 (SrBBS1)

To determine the possible role of the AcBBP1-SrBBS1 protein-DNA interaction in the expression of the *S. rostrata glb3* promoter, we investigated the effect of an insertion mutation in SrBBS1 on *Srglb3* promoter activity using chimeric *gus* reporter genes and transgenic plants as described

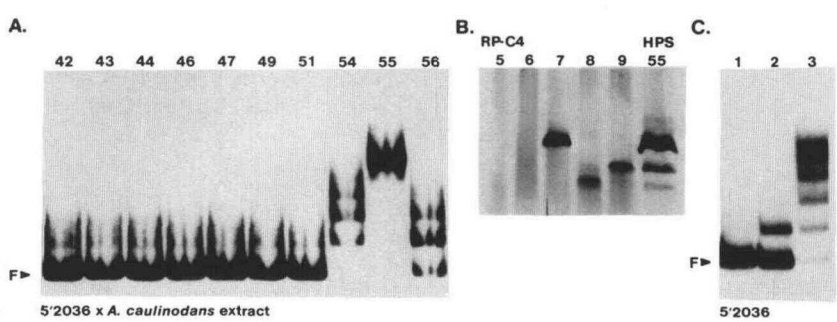

Figure 9. Purification of AcBBP1. A, Retardation gel of fragment 5'2036 after incubation in the presence of different *A. caulinodans* ORS571 culture extract fractions collected from an HPS column. The fraction numbers are indicated above. The multiple complexes formed with fractions 54 to 56 represent complexes formed by single or multiple AcBBP1 molecules to the multiple SrBBS1 sequences carried by fragment 5'2036. B, Photograph of a silver-stained SDS-polyacrylamide gel of HPS fraction 55 and the fractionation products from an RP-C4 column. The fraction numbers (RP-C4 5–9) are indicated above the panel. C, Retardation gel of fragment 5'2036 after incubation in the presence of three fractions collected from a Sephadex G-25 (NAP-10) column, onto which the denatured/renatured protein corresponding to RP-C4 fraction 8 (B) had been loaded. Fraction 3 shows the highest (AcBBP1) binding activity, as evidenced by the formation of multiple complexes (see above). Arrowhead F denotes the free 5'2036 fragment.

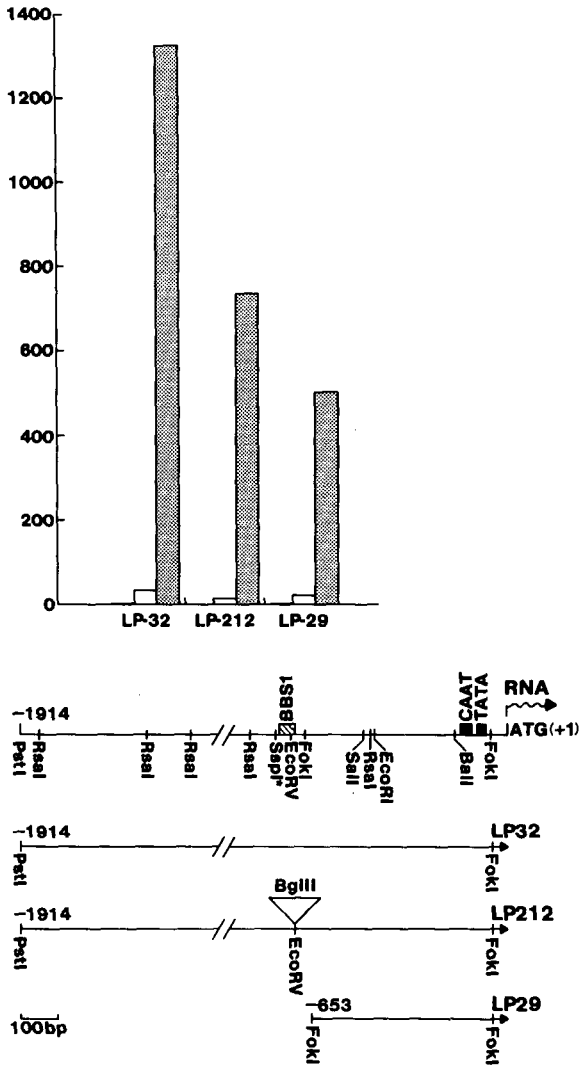

Figure 11. Role of the SrBBS1 region in *Srglb3* promoter activity. The top panel shows the Gus activity of chimeric *Srglb3* promoter-*gus* genes in transgenic *L. corniculatus* plants. Gus activity is expressed in pmol of 4-methylumbelliferone produced min^{-1} mg^{-1} of protein in the extract and represents an average value derived from 10 individual transgenic plants per construct, as described by Szabados et al. (1990). The shaded columns represent Gus expression in nodules, the open columns represent Gus expression in roots, and the short lines to the left of the open columns represent the Gus expression in leaves (baseline expression on this scale). The structure of the chimeric constructs used is shown under the Gus expression panel. The numbers to the left indicate the position of the *PstI* (−1914) and *FokI* (−653) sites, relative to the ATG start codon of the *Srglb3* gene. A scale (in 100 bp) is given. The position of the SrBBS1 is indicated on the first line by a hatched box and the positions of the CAAT and TATA promoter elements by solid boxes. The start point of transcription is indicated by a wavy arrow, labeled RNA. The position of the 40-bp *BglII* linker insertion in the SrBBS1 site is indicated by an open triangle on line 3.

by de Bruijn et al. (1990). A *BglII* linker (40 bp) was inserted into the *EcoRV* site of SrBBS1 (Fig. 1), and a 1.9-kb mutant *Srglb3* promoter fragment (*PstI-FokI*; LP212; Fig. 11) was fused to the β-Gus (*gus*; *uidA*) reporter gene and introduced into *L. corniculatus* plants via *A. rhizogenes*-mediated transformation. Two constructs were used as controls: LP32 (containing the wild-type *PstI-FokI* fragment fused to *gus*) and LP29 (carrying the *FokI-FokI* subfragment of the *Srglb3* promoter, without SrBBS1, fused to *gus*; Szabados et al., 1990; Fig. 11). Gus activity, directed by the chimeric genes, was measured in stems, roots, and nodules of 10 independent transgenic *Lotus* plants. All three constructs (LP32, LP212, and LP29) showed a high degree of nodule specificity in their expression patterns (Fig. 11). However, both the linker insertion in SrBBS1 (LP212) and the unilateral deletion of SrBBS1 (LP29) resulted in an approximately 2-fold decrease of *Srglb3* promoter activity in transgenic plants (Fig. 11).

To relate this in vivo finding back to the in vitro protein-DNA interaction results, we examined the ability of fragment 5'202*, carrying the *BglII* insertion mutation, to interact with AcBBF1. The 40-bp *BglII* linker insertion in the middle of the SrBBS1 was found to result in a significant (50%) reduction of complex formation (data not shown).

DISCUSSION

A/T-rich DNA elements interacting with different nuclear proteins, such as the redundant elements described here for the *S. rostrata glb3* promoter, have been found in the 5' upstream regions of a group of photoregulated genes from pea, tomato, and tobacco (Datta and Cashmore, 1989), seed protein genes activated in developing embryos of soybean (Jofuku et al., 1987; Allen et al., 1989), French bean (Bustos et al., 1989; Riggs et al., 1989) and sunflower (Jordano et al., 1989), the radish rDNA promoter (Echeverria et al., 1992), and soybean heat-shock promoters (Czarnecka et al., 1989). Some of these elements appear to interact with related factors (Jordano et al., 1989; Forde et al., 1990). The A/T-rich regions in these different plant gene promoters have been correlated with (positive) regulatory elements in selected cases (Datta and Cashmore, 1989; Riggs et al., 1989), and a 55-bp oligonucleotide carrying one of these A/T elements has been found to act as a general enhancer in transgenic tobacco plants (Bustos et al., 1989). Thus, the proteins interacting with these A/T elements could belong to a family of general regulatory proteins, involved in controlling expression of PolII-transcribed plant genes (Echeverria et al., 1992).

The A/T-binding site-type sites in the late-nodulin promoters, such as the *Srglb3* promoter analyzed here, have also been associated with positive regulatory regions in several cases (Stougaard et al., 1987; Jensen et al., 1988; de Bruijn et al., 1989; 1990; Szabados et al., 1990; Forde et al., 1990; de Bruijn and Schell, 1992). An oligonucleotide carrying the *S. rostrata glb3* promoter BS2* site has been found to act as a weak enhancer when fused to a minimal plant promoter and introduced into *L. corniculatus* plants. This effect is not nodule specific but, rather, is most pronounced in the roots of the transgenic plants (L. Szabados, P. Welters, and F.J. de Bruijn, unpublished results), as has been observed with the A/T element of the French bean β-phaseolin gene (Bustos et al.,

1989). In addition, selected single-bp mutations in the A/T motif have been found to reduce *S. rostrata glb3* promoter activity significantly in transgenic *L. corniculatus* plants (K. Szczyglowski, S. Y. Fujimoto, L. Szabados, P. Welters, and F.J. de Bruijn, unpublished results). However, in the case of the soybean *lbc3* gene, an internal deletion removing most of the BS1- and BS2-binding sites was found to result in only a slight decrease of promoter activity in transgenic plants (Stougaard et al., 1990).

The significance of the redundancy of the A/T elements in the *S. rostrata glb3* 5′ upstream region and of their specific interaction with multiple DNA-binding proteins from different tissues (including the nodule-specific NAT2 protein) remains to be fully explained. Our *cis* analysis of the *Srglb3* promoter region suggests that they are not absolutely required for nodule-specific expression, because a 78-bp DNA fragment between positions −194 and −116 (relative to the transcription start point), which lacks A/T elements, is sufficient to confer nodule specificity on a heterologous promoter (de Bruijn and Schell, 1992; K. Szczyglowski, S.Y. Fujimoto, L. Szabados, and F.J. de Bruijn, unpublished results). It appears to be more likely that they serve as weak general enhancers, because unidirectional deletions in the *Srglb3* promoter, which progressively remove A/T elements, result in a gradual lowering of gene expression without affecting the tissue specificity of the promoter (Szabados et al., 1990; de Bruijn and Schell, 1992). However, if they are merely general enhancer elements, it is difficult to explain why the A/T elements interact specifically with NAT2, a nodule-specific DNA-binding protein (Jacobsen et al., 1990). Because A/T elements bind to the HMG-like proteins NAT1 and LAT1 (from nodules/roots and young leaves, respectively; Jacobsen et al., 1990; Jensen, 1991), it has been proposed that NAT2 binding may have an effect on chromatin structure in the vicinity of important promoter elements by playing a direct role in nuclear scaffold formation or by interfering with HMG protein binding (Forde et al., 1990). The positions of matrix-associated regions or scaffold-associated regions in the *Srglb3* 5′ upstream region are being mapped relative to the sites containing A/T elements to further investigate this hypothesis.

The SrBBS1-AcBBP1 interaction described here appears to be very different from the A/T element-NAT1, -NAT2, or -LAT1 interactions and is truly unique in various respects. Most important, it suggests the hypothesis that, in the highly evolved endosymbiotic relationship between rhizobia and their host plant, bacterial *trans*-acting factors (proteins) may be involved in modulating the expression of certain plant (nodulin) genes by binding to specific elements in their 5′ upstream regions. Such a mechanism might be useful in the case of those late-nodulin genes, such as the *lb* genes of *S. rostrata*, which appear to be expressed exclusively in the infected cells of the nodule and, therefore, may require the physical presence of bacteroids in the plant cell cytoplasm for their induction (Studer et al., 1987; de Bruijn et al., 1989; Dickstein et al., 1991; de Bruijn and Schell, 1992).

The form of microbe-plant signaling that we are proposing here is not an entirely new idea. An analogous concept was suggested in 1972 by Bruening and Wullstein, who demonstrated that rhizobial metabolites (proteins) could be transferred to the nuclei of clover nodule cells and suggested that "nodulating rhizobia may induce clover nodule cells to initiate leghemoglobin synthesis by the transfer of a bacterially produced inducer." Moreover, Truchet et al. (1980) proposed the existence of a nondiffusible *Rhizobium*-related signal involved in the differentiation of the central (infected) tissue of the developing nodule (central tissue differentiation-inducing principle), and de Billy et al. (1991) suggested that such a factor may, in fact, play a role in infected cell-specific expression of the *lb* genes in alfalfa nodules.

Most of the examples of modulation of plant host (nuclear) gene expression by another organism derive from studies of pathogenic interactions. For example, two polyvirus-encoded proteins (NIa and NIb) are targeted to the nucleus, where they are proposed to be involved in viral RNA replication (Restrepo et al., 1990; Carrington et al., 1991). Bacterial and fungal infection of plant cells results in the induction of a large number of host genes involved in hypersensitive and resistance responses (Lamb et al., 1989; Keen, 1990; Knogge, 1991). The latter mechanisms are especially relevant here, because symbiotic plant-microbe interactions may have evolved from plant-pathogen interactions (Djordjevic et al., 1987).

The chloroplast-nucleus interaction may also provide significant parallels with the symbiotic *Rhizobium*-plant system, because intracellular bacteroids surrounded by a peribacteroid membrane (symbiosomes; Roth et al., 1988) have been considered "organelle like." Evidence is accumulating that shows that specific signal transduction pathways from chloroplasts to the nucleus exist and that they regulate the expression of nuclear genes involved in photosynthesis (Susek and Chory, 1992). Since our initial results suggest the presence of a DNA-binding protein in a bacterium that is transported out of the bacterial cell and into the host plant nucleus, an interesting comparison can be made with the *Agrobacterium*-plant interaction. The transfer of the T-DNA from agrobacteria into the plant cell nucleus has been shown to involve two distinct DNA-binding proteins, VirD2 and VirE2, which are synthesized in the bacteria and facilitate the transfer of the T-DNA complex into the nucleus (Herrera-Estrella et al., 1990; Citovsky et al., 1992). Both VirD2 and VirE2 have been shown to contain bipartite NLSs (Howard et al., 1992; Raikhel, 1992). A comparison of the N-terminal amino acid sequence of AcBBP1 with the NLS motifs compiled by Raikhel (1992) suggests that the basic RK residues at positions 4 and 5, followed by the R-R–K (three of six basic residues) motif starting at position 13 (Fig. 10) could serve the role of a bipartite NLS. However, because AcBBP1 is a small protein (<10 kD) and may be made in considerable quantities in the highly infected plant cells, it may not require a NLS and may enter the nucleus through the nuclear pore simply by diffusion (Garcia-Bustos et al., 1991).

On its way to the nucleus, AcBBP1 would have to traverse two distinct membranes as well, the bacteroid and peribacteroid membranes. The secretion of extracellular proteins by bacteria has been shown to involve cleavable signal peptides or internal signal sequences (Hirst and Welch, 1988). The hydrophobic basic nature of the N terminus of AcBBP1 resembles that of excreted bacterial proteins (Pugsley, 1989). Not much is known about excreted proteins in rhizobia. In

R. leguminosarum bv *viciae*, a secreted, flavonoid-inducible protein (NodO), involved in the symbiotic interaction with host plants, such as *Vicia sativa*, has been identified (de Maagd et al., 1989; Economou et al., 1989, 1990). Secretion of NodO into the culture medium was shown to occur in the absence of N-terminal transit sequence cleavage (Economou et al., 1990), but no data are available about its possible excretion out of the symbiosome in vivo. The NodO protein shares partial amino acid sequence similarity with the *E. coli* hemolysin protein, which is also a secreted protein, binds calcium, and appears to be involved in early stages of plant-microbe interactions (Economou et al., 1990), although its precise function remains to be elucidated.

The N-terminal part of the AcBBP1 protein shares amino acid sequence similarity with a domain of the HSV ICP8 (Gao et al., 1988) protein (46% identity; 68% similarity). ICP8 is a viral protein involved in HSV replication (Conley et al., 1981; Challberg, 1986; Gao et al., 1988) and late gene expression (Gao and Knipe, 1991). In vitro studies have revealed that ICP8 binds to DNA nonspecifically, although it appears to have some preference for single-stranded DNA (Bayliss et al., 1975; Purifoy and Powell, 1976; Lee and Knipe, 1985). ICP8 is also capable of lowering the melting point of poly(dAdT) helices (Powell et al., 1981). The functional significance of the domain of ICP8 sharing a stretch of similar amino acids with AcBBP1 is not known, although mutagenesis experiments have revealed the presence of several other regions in the ICP8 protein that are important for DNA binding and regions that act synergistically or antagonistically in nuclear targeting (Orberg and Schaffer, 1987; Gao and Knipe, 1989, 1992). Therefore, it is difficult to clearly assign a functional significance to the observed similarities, and further analyses will need to await the determination of the complete sequence of the *A. caulinodans* gene encoding AcBBP1.

The evidence presented here does not allow us to conclude that the *A. caulinodans* protein we have found to interact specifically with the *S. rostrata glb3* promoter (AcBBP1) indeed corresponds to a *trans*-acting regulatory factor involved in infected cell-specific expression of *lb* genes. On the one hand, the developmental appearance of the SrBBS1-AcBBP1 complex 12 d after infection, concomitantly with the onset of Lb production, is consistent with a role for AcBBP1 in *lb* gene expression. On the other hand, its target site, SrBBS1, is not strictly required for nodule infected cell-specific expression, because a chimeric *Srglb3-gus* construct, carrying the first 429 bp of the *Srglb3* 5′ upstream region (excluding the SrBBS1), shows a nodule-specific expression pattern, although at a reduced level (LP31; Szabados et al., 1990). We note that the SrBBS1 does appear to be important for a high level of nodule (infected cell-specific) expression, because an insertion mutation in SrBBS1 leads to a 50% reduction of gene expression. The same mutation results in a 50% reduction of AcBBF1 binding, suggesting a link between *trans*-acting factor binding and action in *cis*. The SrBBS1 sequence motif is conserved at the analogous position (approximately −700) of the *S. rostrata glb2* 5′ upstream region (21 of 25 bp conserved) and shares 16 of 25 bp similarity with a motif about 1000 bp upstream of a pea *lb* 5′ upstream region (Nap, 1988). No data are available about the functional role of the homologous elements. Final proof of the AcBBP1 involvement in *lb* gene expression will require analysis of an *A. caulinodans* mutant strain deficient in BBP1 synthesis, which was created via reverse genetics. This experiment is in progress.

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LITERATURE CITED

Allen RD, Bernier F, Lessard PA, Beachy RN (1989) Nuclear factors interact with a soybean β-conglycinin enhancer. Plant Cell **1:** 623–631

Bayliss GJ, Marsden HS, Hay J (1975) Herpes simplex virus proteins: DNA-binding proteins in infected cells and in the virus structure. Virology **68:** 124–134

Beringer JE (1974) R factor transfer in *Rhizobium leguminosarum*. J Gen Micobiol **84:** 188–189

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Breining P, Piepersberg W (1986) Yeast omnipotent suppressor *SUP1* (*SUP45*): nucleotide sequence of the wildtype and a mutant gene. Nucleic Acids Res **14:** 5187–5197

Bruening ML, Wullstein LH (1972) Evidence for the transfer of rhizobial metabolites to the polyploid nuclei of young clover roots. Physiol Plant **27:** 244–252

Bustos MM, Guiltinan MJ, Jordano J, Begum D, Kalkan FA, Hall TC (1989) Regulation of β-glucuronidase expression in transgenic tobacco plants by an A/T-rich, *cis*-acting sequence found upstream of a French bean β-phaseolin gene. Plant Cell **1:** 839–853

Carrington JC, Freed DD, Leinicke AJ (1991) Bipartite signal sequence mediates nuclear translocation of the plant polyviral N1A protein. Plant Cell **3:** 953–962

Challberg MD (1986) A method for identifying the viral genes required for herpesvirus DNA replication. Proc Natl Acad Sci USA **83:** 9094–9098

Citovsky V, Zupan J, Warnick D, Zambryski P (1992) Nuclear localization of *Agrobacterium* VirE2 protein in plant cells. Science **256:** 1802–1804

Conley AJ, Knipe DM, Jones PC, Roizman B (1981) Molecular genetics of herpes simplex virus. VII. Characterization of a temperature-sensitive mutant produced by in vitro mutagenesis and defective in DNA synthesis and accumulation of polypeptides. J Virol **37:** 413–428

Czarnecka E, Key JL, Gurley WB (1989) Regulatory domains of the *Gmhsp*17.5-E heat shock promotor of soybean. Mol Cell Biol **9:** 3457–3463

Datta N, Cashmore AR (1989) Binding of a pea nuclear protein to promoters of certain photoregulated genes is modulated by phosphorylation. Plant Cell **1:** 1069–1077

de Billy F, Barker DG, Gallusci P, Truchet G (1991) Leghaemoglobin gene transcription is triggered in a single cell layer of the indeterminate nitrogen-fixing root nodule of alfalfa. Plant J **1:** 27–35

de Bruijn FJ, Downie JA (1991) Biochemical and molecular studies of symbiotic nitrogen fixation. Curr Opin Biotechnol **2:** 184–192

de Bruijn FJ, Felix G, Grunenberg B, Hoffmann HJ, Metz B, Ratet

P, Simons-Schreier A, Szabados L, Welters P, Schell J (1989) Regulation of plant genes specifically induced in nitrogen-fixing nodules: role of *cis*-acting elements and *trans*-acting factors in leghemoglobin gene expression. Plant Mol Biol **13:** 319–325

de Bruijn FJ, Jensen EØ, Metz BA, Hoffmann HJ, Welters P, Marcker KA, Schell J (1988) Interaction of *trans*-acting factors with distinct DNA elements in the promoter regions of the *Glycine max* and *Sesbania rostrata* leghemoglobin genes. *In* R Palacios, DPS Verma, eds, Molecular Genetics of Plant-Microbe Interactions. APS Press, St. Paul, MN, pp 333–339

de Bruijn F J, Schell J (1992) Regulation of plant genes specifically induced in developing and mature nitrogen-fixing nodules: *cis*-acting elements and *trans*-acting factors. *In* DPS Verma, ed, Control of Plant Gene Expression. CRC Press, Boca Raton, FL, pp 241–258

de Bruijn FJ, Szabados L, Schell J (1990) Chimeric genes and transgenic plants are used to study the regulation of genes involved in plant-microbe interactions (nodulin genes). Dev Genet **11:** 182–196

de Maagd RA, Spaink HP, Pees E, Mulders IHM, Wijfjes A, Wijffelman CA, Okker R, Lugtenberg BJJ (1989) Localization and symbiotic function of a region on the *Rhizobium leguminosarum* Sym plasmid pRL1JI responsible for a secreted, flavonoid-inducible 50-kilodalton protein. J Bacteriol **171:** 1151–1157

Dehio C, de Bruijn FJ (1992) The early nodulin *Sr*Enod2 from *Sesbania rostrata* is inducible by cytokinin. Plant J **2:** 117–128

Dickstein R, Scheirer DC, Fowle WH, Ausubel FM (1991) Nodules elicited by *Rhizobium meliloti heme* mutants are arrested at an early stage of development. Mol Gen Genet **230:** 423–432

Djordjevic MA, Gabriel DW, Rolfe BG (1987) *Rhizobium*—the refined parasite of legumes. Annu Rev Phytopathol **41:** 339–367

Dreyfus B, Garcia JL, Gillis M (1988) Characterization of *Azorhizobium caulinodans* gen. nov. sp. nov., a stem-nodulating nitrogen-fixing bacterium isolated from *Sesbania rostrata.* Int J Syst Bacteriol **38:** 89–98

Echeverria M, Delcasso-Tremousaygue D, Delseny M (1992) A nuclear protein binding to dA/dT-rich sequences upstream from the radish DNA promoter. Plant J **2:** 211–219

Economou A, Hamilton WDO, Johnston, AWB, Downie JA (1990) The *Rhizobium* nodulation gene *nodO* encodes a Ca^{2+} binding protein that is exported without N-terminal cleavage and is homologous to haemolysin and related proteins. EMBO J **9:** 349–354

Economou A, Hawkins FKL, Downie JA, Johnston AWB (1989) Transcription of *rhiA*, a gene on a *Rhizobium leguminosarum* bv. *viciae* Sym plasmid, requires *rhiR* and is repressed by flavanoids that induce *nod* genes. Mol Microbiol **3:** 87–93

Felix G (1989) Expression of leghemoglobin and Enod2 genes in stem nodules of *Sesbania rostrata* induced by *Azorhizobium caulinodans* ORS571 wildtype and mutant strains. Masters thesis. Max Planck Institut, Cologne, Germany

Forde BG, Freeman J, Oliver JE, Pineda M (1990) Nuclear factors interact with conserved A/T-rich elements upstream of a nodule-enhanced glutamine synthetase gene from French bean. Plant Cell **2:** 925–939

Gao M, Bouchey J, Curtin K, Knipe DM (1988) Genetic identification of a portion of the herpes simplex virus ICP8 protein required for DNA-binding. Virology **163:** 319–329

Gao M, Knipe DM (1989) Genetic evidence for multiple nuclear functions of herpes simplex virus ICP8 protein. J Virol **63:** 5258–5267

Gao M, Knipe DM (1991) Potential role for herpes simplex virus DNA replication protein in stimulation of late gene expression. J Virol **65:** 2666–2675

Gao M, Knipe DM (1992) Distal protein sequences can affect the function of a nuclear localization signal. Mol Cell Biol **12:** 1330–1339

Garcia-Bustos J, Heitman J, Hall MN (1991) Nuclear protein localization. Biochim Biophys Acta **1071:** 83–101

Goodman LE, Perou CM, Tamanoi F (1988) Structure and expression of yeast DPRI, a gene essential for the processing and intracellular localization of Ras protein. Yeast **4:** 271–281

Hansen J, Joergensen JE, Stougaard J, Marcker KA (1989) Hairy roots—a short cut to transgenic root nodules. Plant Cell Rep **8:** 12–15

Herrera-Estrella A, Van Montagu M, Wang K (1990) A bacterial peptide acting as a plant nuclear targetting signal: the amino-terminal portion of *Agrobacterium* VirD2 protein directs a β-galactosidase fusion protein into tobacco nuclei. Proc Natl Acad Sci USA **87:** 9534–9537

Hirst TR, Welch RA (1988) Mechanisms for secretion of extracellular proteins by gram-negative bacteria. Trends Biochem Sci **13:** 265–269

Hotter GS, Scott DB (1991) Exopolysaccharide mutants of *Rhizobium loti* are fully effective on a determinate nodulating host but are ineffective on an indeterminate nodulating host. J Bacteriol **173:** 851–859

Howard EA, Zupan JR, Citovsky V, Zambryski P (1992) The VirD2 protein of *A. tumefaciens* contains a C-terminal bipartite nuclear localization signal: implications for nuclear uptake of DNA in plant cells. Cell **68:** 109–118

Jacobsen K, Laursen NB, Jensen EO, Marcker A, Poulsen C, Marcker KA (1990) HMG I-like proteins from leaf and nodule nuclei interact with different AT motifs in soybean nodulin promoters. Plant Cell **2:** 85–94

Jensen EO (1991) Regulation of nodule-expressed soybean genes. *In* H Hennecke, DPS Verma, eds, Advances in Molecular Genetics of Plant-Microbe Interactions, Vol 1. Kluwer Academic, The Hague, The Netherlands, pp 310–316

Jensen EO, Marcker KA, Schell J, de Bruijn FJ (1988) Interaction of a nodule specific, *trans*-acting factor with distinct DNA elements in the soybean leghemoglobin 5′ upstream region. EMBO J **7:** 1265–1271

Jofuku KD, Okamura JK, Goldberg RB (1987) Interaction of an embryo DNA binding protein with a soybean lectin gene upstream region. Nature **328:** 738–740

Jordano J, Almoguera C, Thomas T (1989) A sunflower heliathinin gene upstream sequence ensemble contains an enhancer and sites of nuclear protein interaction. Plant Cell **1:** 855–866

Keen NT (1990) Gene-for-gene complementarity in plant-pathogen interactions. Annu Rev Genet **24:** 447–463

Knogge W (1991) Plant resistance genes for fungal pathogens-physiological models and identificationin cereal crops. Z. Naturforsch. **46c:** 969–981

Lamb CJ, Lawton MA, Dron M, Dixon RA (1989) Signals and transduction mechanisms of activation of plant defenses against microbial attack. Cell **56:** 215–224

Lee CK, Knipe DM (1985) Immunoassay for the DNA-binding activities of herpes simplex virus protein ICP8. J Virol **54:** 731–738

Long SR (1989) *Rhizobium*-legume nodulation: life together in the underground. Cell **56:** 203–214

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Meade HM, Long SR, Ruvkun GB, Brown SE, Ausubel FM (1982) Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn5 mutagenesis. J Bacteriol **149:** 114–122

Metz BA, Welters P, Hoffmann HJ, Jensen EO, Schell J, de Bruijn FJ (1988) Primary structure and promoter analysis of leghemoglobin genes of the stem nodulated tropical legume *Sesbania rostrata*: conserved coding sequences, *cis*-elements and *trans*-acting factors. Mol Gen Genet **214:** 181–191

Miller JH (1972) Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Nap JP (1988) Nodulins in root nodule development. PhD thesis. Agricultural University, Wageningen, The Netherlands

Nap J, Bisseling T (1990) Development biology of a plant-prokaryote symbiosis: the legume root nodule. Science **250:** 948–954

Orberg PK, Schaffer PA (1987) Expression of herpes simplex virus type I major DNA-binding protein, ICP8, in transformed cell lines: complementation of deletion mutants and inhibition of wild-type virus. J Virol **61:** 1136–1146

Pawlowski K, Ratet P, Schell J, de Bruijn FJ (1987) Cloning and characterization of *nifA* and *ntrC* genes of the stem-nodulating bacterium ORS571, the nitrogen fixing symbiont of *Sesbania ros-*

trata: regulation of nitrogen fixation (*nif*) genes in the free-living versus symbiotic state. Mol Gen Genet **206:** 207–219

Powell KL, Littler E, Purifoy DJM (1981) Nonstructural proteins of herpes simplex virus. II. Major virus-specific DNA-binding protein. J Virol **39:** 894–902

Pugsley AP (1989) Protein Targeting. Academic Press, San Diego, CA

Purifoy DJM, Powell KL (1976) DNA-binding proteins induced by herpes simplex virus type 2 in HEp-2 cells. J Virol **19:** 717–731

Raikhel N (1992) Nuclear targeting in plants. Plant Physiol **100:** 1627–1632

Restrepo MA, Freed DD, Carrington JC (1990) Nuclear transport of plant potyviral proteins. Plant Cell **2:** 987–998

Riggs CD, Voelker TA, Chrispeels MJ (1989) Cotyledon nuclear proteins bind to DNA fragments harboring regulatory elements of phytohemagglutinin genes. Plant Cell **1:** 609–621

Roth LE, Jeon K, Stacey G (1988) Homology in endosymbiotic systems: the term "symbiosome." *In* R Palacios, DPS Verma, eds, Molecular Genetics of Plant-Microbe Interactions, APS Press, St. Paul, MN, pp 220–225

Sanchez F, Padilla JE, Perez H, Lara M (1991) Control of nodulin genes in root-nodule development and metabolism. Annu Rev Plant Physiol Plant Mol Biol **42:** 507–528

Stougaard J, Jørgensen JE, Christensen T, Kuhle A, Marcker KA (1990) Interdependence and nodule specificity of *cis*-acting regulatory elements in the soybean leghemoglobin *lbc3* and *N23* gene promoters. Mol Gen Genet **220:** 353–360

Stougaard J, Sandal NN, Gron A, Kuhle A, Marcker KA (1987) 5′ analysis of the soybean leghaemoglobin *lbc3* gene: regulatory elements for promoter activity and organ specificity. EMBO J **6:** 3565–3569

Studer D, Gloudemans T, Franssen HJ, Fischer HM, Bisseling T, Hennecke H (1987) Involvement of the bacterial nitrogen fixation regulatory gene (*nifA*) in control of nodule-specific host-plant gene expression. Eur J Cell Biol **45:** 177–184

Susek RE, Chory J (1992) A tale of two genomes: role of a chloroplast signal in coordinating nuclear and plastid genome expression. Aust J Plant Physiol **19:** 387–399

Szabados L, Ratet P, Grunenberg B, de Bruijn FJ (1990) Functional analysis of the *Sesbania rostrata* leghemoglobin *glb3* gene 5′-upstream region in transgenic *Lotus corniculatus* and *Nicotiana tabacum* plants. Plant Cell **2:** 973–986

Truchet G, Michel M, Denarie J (1980) Sequential analysis of the organogenesis of lucerne (*Medicago sativa*) root nodules using symbiotically defective mutants of *Rhizobium meliloti*. Differentiation **16:** 163–172

Van Kammen A (1984) Suggested nomenclature for plant genes involved in nodulation and symbiosis. Plant Mol Biol Rep **2:** 43–45

Vance CP, Heichel GH (1991) Carbon in N_2 fixation: limitation or exquisite adaptation. Annu Rev Plant Physiol Plant Mol Biol **42:** 373–392

Verma DPS (1992) Signals in root nodule organogenesis and endocytosis of *Rhizobium*. Plant Cell **4:** 373–382

Verma DPS, Delauney AJ (1988) Root nodule symbiosis: nodulins and nodulin genes. *In* DPS Verma, R Goldberg, eds, Plant Gene Research: Temporal and Spatial Regulation of Plant Genes. Springer Verlag, New York, pp 169–199

Welters P, Felix G, de Bruijn FJ (1990) Specific interactions of a rhizobial protein (*trans*-acting factor) with distinct DNA sequences in the promoter region of a *Sesbania rostrata* leghemoglobin gene. *In* P Gresshoff, E Roth, G Stacey, WE Newton, eds, Nitrogen Fixation: Achievements and Objectives. Chapman and Hall, New York, p 775

Welters P, Metz BA, Schell J, de Bruijn FJ (1989) Nucleotide sequence of a *Sesbania rostrata* leghemoglobin gene. Nucleic Acids Res **17:** 1253

Yanish-Perron C, Viera J, Messing J (1985) Improved M13 cloning vectors and host strains: nucleotide sequence of the M13 mp18 and pUC19 vectors. Gene **33:** 103–110